1994

Permanent fixation of an I/d-Spm insertion

Vijayabhaskar Reddy Thatiparthi
Iowa State University

Order Number 9518448

Permanent fixation of an *i/d-Spm* insertion

Thatiparthi, Vijayabhaskar Reddy, Ph.D.

Iowa State University, 1994

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Permanent fixation of an *I/d-Spm* insertion

by

Vijayabhaskar Reddy Thatiparthi

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Zoology and Genetics
Interdepartmental Major: Genetics

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Interdepartmental Major

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1994

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Transposable elements are the mobile genetic entities of most genomes. Since their first discovery in maize (McClintock, 1948) transposable elements are found in many different organisms, both prokaryotic and eukaryotic (Berg and Howe, 1989). Transposition or movement of these elements is brought about by the binding of a protein called transposase (trans-acting factor) that is encoded by the element itself to the subterminal sequence motifs of the element (cis-determinants of transposition; Peterson, 1987; Fedoroff, 1989b; Gierl et al., 1989). Elements which can transpose on their own are called autonomous elements and those which can not transpose on their own are called non-autonomous elements (Fincham and Sastry, 1974). Non-autonomous elements are deletion derivatives of autonomous elements (Fedoroff et al., 1983; Fedoroff, 1989b; Gierl et al, 1989; Walbot 1991; Bennetzen et al., 1993). The autonomous and their derivative non-autonomous elements are the two major components of any transposable element family (Peterson, 1987). However, a third component namely *mediator* is shown to be required, in one case, for transposition (Muszynski et al., 1993).

In a majority of the genomes there are several copies of the transposable elements and in most cases these elements are passive constituents of these genomes (Berg and Howe, 1989). However, these quiescent elements can get activated when genomes are subjected to severe stress. Exposure to natural agents like viruses as well as the artificial irradiation can inflict such a stress on most genomes (McClintock, 1965b; Sprague and McKinney, 1966; Dellaporta et al, 1984b; Peterson, 1985b). The activated elements, through their transposition activity, can bring about a wide variety of changes in genome

organization (McClintock, 1984). Such changes may vary from gross chromosomal rearrangements to simple nucleotide mutations (Nevers and Saedler, 1977; Walbot and Cullis, 1985). Two major implications of these changes are: increased genomic stress and creation of new genetic variation (i.e., altered gene expression). The newly created genetic variation is thought to be useful in the evolution of organisms (Schwarz-Sommer et al., 1985b; Wessler, 1988). However, most organisms have developed mechanisms of silencing these elements via methylation in order to protect themselves from the deleterious effects of excessive transposition (Fedoroff, 1989b). Apart from their role in genome reorganization transposable elements are widely recognised for their usefulness in the isolation of genes by transposon tagging.

The maize genome is the home of a variety of transposable elements (Peterson, 1987). In the past five decades several transposable elements were discovered in maize and among them the *Ac/Ds* system (McClintock, 1948), *En/Spm* system (Peterson, 1953, 1961; McClintock, 1954) and the *Mutator* system (Robertson, 1978) have been extensively studied. A detailed survey of tribal maize and the maize lines from breeders' nurseries suggested that these elements are widely distributed (Gonella and Peterson, 1975; Peterson and Salamini, 1986; Cormack et al., 1988; Lamkey et al., 1991). Their pervasiveness in the breeding lines suggested that part of the genetic variation might be transposable element generated (Gierl et al., 1989).

The footprints (few nucleotide additions at the site of insertion) generated upon element excision are thought to be the major source of new genetic variation (Schwarz-Sommer et al., 1985b; Wessler, 1988). However, a subset of *I* element (non-autonomous element of *En/Spm* system) insertions are also capable of creating genetic variation not

through footprints but by being stably inserted in a particular gene. These elements are never observed to excise from their host genes even in the presence of active autonomous *En/Spm* elements. Loss of the cis-determinants and the smaller size of the elements are thought to be the main causes that render these inserts stable (Menssen et al, 1990; Aukerman and Schmidt, 1993; Bunkers et al., 1993). Some of these inserts also behave like `introns' (Raboy et al., 1989; Menssen et al., 1990; Bunkers et al., 1993). Introns are known to increase gene expression in certain cases (Callis el al., 1987; McElroy et al., 1990; Leuhersen and Walbot, 1991). Therefore some of these inserts have the potential of creating new genetic variation. However, these stable inserts continue to interact with the transposases encoded by the autonomous *En/Spm* element. These autonomous element encoded transposases are thought to be responsible for causing deletions within the element (McClintock, 1965b; Fedoroff, 1989b; Gierl et al., 1989). Therefore this continued interaction of the *I* element with the transposase casts some doubt on their stability. There is a dearth of available data on the extent of stability of these *I* elements in the presence of the autonomous *En/Spm* element.

In order to gain an understanding of the extent of stability of these inserts we undertook a study of the *I* element of *a2-m1*(class II state) allele (McClintock, 1958). The *I* element of this allele is not only stably inserted (McClintock, 1957, 1958, 1971) but also acts as an intron in an otherwise intronless *A2* gene (Menssen et al., 1990). This *I* element also continues to interact with the TNPA product of the *En/Spm* element. Thus the *a2-m1*(class II state) allele is an ideal experimental material to study the extent of stability of the *I* element in the presence of an active *En/Spm* element. The results of this experiment along with the identification and genetic characterization of a dominant

modifier of *En/Spm* element are presented in this thesis. Since the stable inserts of the type present at *a2-m1* allele have the potential of creating genetic variation, the results of our experiments would help to gain an insight on the extent of stability of such newly created genetic variation as well as the extent of stability of newly acquired introns.

2. LITERATURE REVIEW

Maize transposable elements are among the most well characterized transposable elements of the eukaryotes. In the past few decades extensive research both genetic as well as molecular has investigated the various transposable element systems in maize. In this chapter the literature that is pertinent only to the *En/Spm* transposable element system is reviewed . Relevant work from other transposable elements also is included wherever it is deemed necessary.

2.1. Discovery of Transposable Elements

2.1.1. Variegation in plants

In plants some of the vegetative and reproductive structures are often brightly colored ranging from the deep red to crimson, purple and yellow. These colors are imparted by a group of plant pigments called flavonoids (Goodwin and Mercer, 1983). Apart from the uniformly colored plant parts one can often see a mixture of colors in various patterns. This mosaic pattern of colors is termed variegation (Kirk and Tilney-Basset, 1978). The variegated phenotype can also be observed in various plant parts particularly in leaves, flowers and seeds.

Shortly after the rediscovery of Mendel's laws of inheritance in 1900's many scientists turned their attention to studying the inheritance of variegation pattern in plants. It was soon discovered that variegation can be caused both by Mendelian as well as non-Mendelian factors (Evenari, 1989).

The non-Mendelian factors include disease induced variegation as for example leaf chlorosis developed after viral infection (Evenari, 1989) and maternal inheritance. The flower variegation in *Mirabilis jalapa* was the first reported case of maternal inheritance (by Correns, reviewed in Evenari,1989) where the maternal flower traits determine the variegation pattern. Some environmental factors such as light and temperature can also alter, though not heritably, the expression of variegation phenotype (Evenari, 1989).

In a majority of cases, the variegation is inherited in Mendelian fashion. The white-green variegation in leaves may be caused by dominant, semidominant or recessive nuclear genes (Evenari, 1989). The discovery of these nuclear genes has led to the emergence of some interesting concepts such as 'unstable or mutable genes' (reviewed in Peterson, 1987). Perhaps the most significant of them all was the discovery made by Barbara McClintock in late 1940's who proposed that certain types of kernel variegation in maize are caused by 'distinct genetic elements' capable of moving from one place to the other in the maize genome.

2.1.2. McClintock's pioneering work in maize

The first reports of kernel variegation in maize are the 'variegated pericarp' (Emerson, 1917) and the kernel spotting phenotype called 'dotted' (Rhoades, 1936, 1938). While the variegation of pericarp is controlled by a single gene (*P-rr*) the dotted phenotype is the result of the interaction between the recessive *a1* gene and an independently segregating dominant factor which Rhoades called *Dt* (dotted; Rhoades,1938). However, the true nature of such kernel variegation was revealed only after the discovery of 'controlling elements' in maize by McClintock.

McClintock's initial work on broken chromosomes led her to the discovery of the phenomenon of 'breakage-fusion-bridge' (BFB) cycle (McClintock, 1938, 1939, 1941 and 1942). BFB cycle results when the sister chromatids of a broken chromosome fuse at their broken ends forming a bridge at anaphase which in turn breaks while the two chromatids move to the opposite poles (McClintock, 1941). The chromosome that was investigated was fortunately chromosome 9 which carries several kernel specific genes on its short arm including two of the color determining genes *C* and *Bz*. When a chromosome 9 containing dominant color genes undergoes BFB cycle in a kernel heterozygous for color determining genes, variegated kernel phenotypes result instead of the normal colored phenotype (McClintock, 1941).

The chromosome breakage induced by BFB occurs at random sites on the chromatid bridge. However, McClintock later observed the occurrence of chromosome breakage at a precise position proximal to *the Wx* locus on chromosome 9. This locus was termed *'Dissociation (Ds)'* (McClintock, 1946). Subsequently McClintock found that the breakage at *the Ds* locus occurs only in the presence of another independently segregating factor called *'Activator (Ac)'* (McClintock, 1947). It was soon revealed that the position of *Ds* in the genome was not fixed. *Ds* appears to occupy different positions within the short arm of chromosome 9. The appearance of *Ds* at the new locus coincided with the loss of *Ds* activity at the original locus (McClintock, 1948). This phenomenon whereby the *Ds* locus moves from one position to other in the genome in the presence of *Ac* was termed as transposition by McClintock (1949). Thus the concept of 'transposable elements' came into being in the realm of genetics (reviewed in Peterson, 1987).

Full recognition to McClintock's discovery of transposable elements came only after the finding and molecular isolation of similar elements in bacteria i.e., phage *Mu* and *IS* (insertion sequences) elements (Taylor, 1963; Jordan et al., 1964; Saedler and Starlinger, 1967; Adhya and Shapiro, 1969; Peterson, 1970b; Nevers and Saedler, 1977). Soon transposable elements were discovered in many other organisms both prokaryotic and eukaryotic (Berg and Howe, 1989).

2.2. General Features of Transposable Elements

2.2.1. Genetic traits

Although transposable elements were discovered in many organisms they are well characterized genetically only in few organisms such as bacteria (Galas and Chandler, 1989), *Drosophila* (Engels, 1989), yeast (Boeke, 1989), maize (Peterson, 1987; Fedoroff, 1989b) and *Antirrhinum* (Coen et al., 1989). The various transposable element systems present in these organisms, though differing from each other in some aspects, share certain common genetic features. However, only the features of maize transposable elements are presented here.

The unique feature of transposable elements is their ability to move within the genome (McClintock, 1948). Those elements which are capable of transposing on their own are called autonomous (Fincham and Sastry, 1974) or regulatory (McClintock, 1961) elements. Those elements which can not transpose on their own and depend on the regulator element for their transposition are termed non-autonomous or receptor elements

(Fincham and Sastry, 1974). Most of the elements characterized thus far are composed of both autonomous and non-autonomous elements.

Transposable elements often are inherited in a Mendelian fashion. In general these elements exist in a hemizygous condition and when test crossed, autonomous elements give rise to segregation ratios typical of a heterozygous condition i.e., 1 mutable : 1 normal (wild type or recessive as the case may be) phenotypes. However, in certain cases they may exist in homozygous condition (McClintock, 1954; Friedeman and Peterson, 1982; Peterson, 1990).

The process of transposition involves two main events - insertion of transposons into genes and excision of the same from those genes. Insertion leads to a loss of gene function whereas excision restores gene function at various rates. These insertion and excision events when occurring in somatic tissue result in a mutable or variegated phenotype. The recurrence of such mutable phenotypes is a diagnostic feature of the presence of transposable elements in the genome.

When excisions occur in germinal tissue that give rise to male and female gametes they result in reversion of the mutable phenotype to wild type phenotype. The degree of expression of the reverted gene depends on the position of the insertion within the gene. Since the excision of transposons is imprecise in maize (Schwarz-Sommer et al., 1985b; Baran et al., 1992), certain regions of the gene (for example introns) are more tolerant to the addition of extra nucleotides (resulting from the duplication of short stretches of nucleotides of the host gene upon element insertion) than others (for example promoters and exons). These extra nucleotides that are left behind following element excision are termed 'footprints'. A wide variety of revertant phenotypes ranging fron recessive to wild

type were reported for *A1* (Peterson, 1961, 1970a), *A2* and *C1* (Reddy and Peterson, 1976). The reversion frequencies vary from gene to gene and in particular from system to system. For example, while the occurrence of revertants is a common event with *Ac/Ds* and *En(Spm)/I(d-Spm)* elements, it is rare with *Mu* transposable element (Walbot, 1991).

Another genetic feature of transposable elements is that their transposition activity results in the increase of spontaneous mutation rate of a particular gene by several fold. This mutation rate may vary from as low as 8.2×10^{-6} (Nelson and Klein, 1984) to as high as 2×10^{-2} (McClintock, 1952). Frequencies vary from gene to gene and from system to system (reviewed in Döring, 1989). Since some of the elements preferably transpose to closely linked sites (van Schaik and Brink, 1959; Nowick and Peterson, 1981; Dooner and Belachew, 1989), the close proximity of a certain transposable element to a gene will further increase the mutation rate of that particular gene (Döring, 1989). This unique feature of transposable elements is exploited in gene tagging and in the development of novel genetic stocks where all the chromosomal arms of maize were labelled with *En/Spm* autonomous transposable element (Dash and Peterson, 1989; Chang and Peterson, 1994). In these stocks, an autonomous element is introduced into each chromosome arm via chromosomal translocations which brings the element in close proximity to the genes present in that arm and thereby increase the probability of tagging those genes.

2.2.2. Molecular traits

Apart from sharing some genetic traits transposable elements also share some common molecular features (Peterson, 1987; Fedoroff, 1989b; Gierl et al., 1989; Walbot, 1991; Bennetzen et al., 1993).

In general most of the transposable elements known to date exist in high copy number (50 to 100) in the genome (Gierl, 1990). Within a particular system these copies represent elements of various length. Most of these are deletion derivatives (and hence non-autonomous) of the autonomous elements. Among these some cryptic elements which are of full length (like autonomous elements but non-functional) can also be found (Fedoroff et al., 1983; Periera et al., 1985; Nevers et al., 1986; Gierl, 1990).

One of the consistent features of the transposable elements is the presence of **'terminal inverted regions (TIRs)'** where a short stretch of sequence at one end is repeated in reverse orientation at the other end of the element (Fig. 2.1). The length of these TIRs varies from element to element. The subterminal sequences close to the TIRs at both ends are composed of short repetitive sequences which serve as the binding sites for transposase. These **'subterminal repetitive regions (SRRs)'** together with TIRs serve as the cis-determinants of transposition (Fig. 2.1). The coding region of the transposon is composed of exons and introns like any other gene and one or two open reading frames (ORFs) which encode the transacting protein products commonly termed as **'transposase'**. Some transposable elements like *Ac* encode a single protein product and others like

Figure 2.1. General structure of a transposable element. The target site in the host gene is indicated as a duplicated sequence ATT. The filled arrows are the TIRs. Open boxes are the SRRs. The small arrows within the open boxes represent transposase binding motifs. Filled boxes are the transposase encoding exons.

En/Spm encode two protein products. While autonomous elements are capable of encoding the transposase, the non-autonomous elements, which are the deletion derivatives of autonomous elements, do not encode any protein products. Each transposable element has a specific host sequence, which varies in length from element to element, as its **target site**. The target site will be duplicated upon transposable elment insertion which may be retained in full or in part after element excision (Fig. 2.1; reviewed in Peterson, 1987; Fedoroff, 1989b; Gierl et al., 1989; Walbot, 1991).

2.3. Transposable Element Systems in Maize

2.3.1. Basis of classification of systems

Soon after the discovery of *Ds* and *Ac* transposable element system McClintock found other mutable loci that included *c-m1, c-m2, wx-m1* etc., which originated by the insertion of *Ds* at the *C* and *Wx* loci respectively. The *Ds* elements at these loci are different from the original *Ds* in that it does not cause chromosome breakage. But like the original *Ds*, the *Ds* at these loci require *Ac* in order to show mutability. Based on this interaction between *Ac* and different types of *Ds*, McClintock called this group of elements as '*Ac-Ds* controlling element system' (McClintock, 1948, 1951). The regulator of this system i.e., *Ac* does not trigger mutability at the Rhoades' *a1* locus which is responsive to *Dt* (Rhoades, 1938; McClintock, 1951). Thus the *a1-dt/Dt* group is distinct from *Ac/Ds* and comprises a new controlling element system. Later two other transposable elements namely *Modulator* (*Mp*; Brink and Nilan, 1952) and *Enhancer* (*En* ; Peterson, 1953, 1960) were independently discovered. By using the receptor elements of *Ac* and *En* respectively

as 'testers' the transposable element *Mp* was shown to be analogous to *Ac* (Barklay and Brink, 1957) and the *Suppressor-Mutator* element (*Spm*; McClintock, 1954) was shown to be analogous to *En* (Peterson, 1965). Thus based on the specificity of genetic interaction among the members of these elements, different transposable element **systems** or **families** were established in maize (reviewed in Peterson, 1987). Genetically the term 'system' can now be defined as the group of elements, comprising both autonomous and non-autonomous elements, which interact with members of its own system and not with those of the other systems.

The true nature of the 'specificity of interaction' among the members of various systems was revealed only after the molecular characterization of some of these trasposable elements. The molecular analysis revealed that the specificity determining factors are the transposase binding motifs present in the subterminal region on either end of the element (i.e., SRRs) and TIRs. The number of these binding motifs vary from element to element (Schwarz-Sommer et al., 1984; Periera et al., 1985; Kunze and Starlinger, 1989). These systems also differ in the number of transacting factors (one or two proteins), the length and sequence of their TIRs and finally in their target site selection (refer Table 2.1).

2.3.2. Summary of the systems identified in maize

The various transposable elements that are identified in maize thusfar are summarized in Table 2.1. All the elements present in one box are analogous and thus constitute a system. The *Ac*, *En/Spm* and *Mu* systems are the most extensively studied transposable elements in maize and little is known about the rest of the systems. The molecular identity of the regulatory elements *Uq*, *Dt* and *Mrh* are yet to be revealed. Only

Table 2.1. Transposable element systems in maize

Transposable element system			length of		REFERENCES
#	Regulator	Receptor	TSD[a] (bp)	TIR[b] (bp)	
I.	*Ac*	*Ds*	8	11	McClintock, 1948; Fedoroff et. al., 1983
	Mp	--	--	--	Brink and Nilan, 1957
	Uq	*r-uq*	8	11	Friedeman and Peterson, 1982
					Pisabarro et al., 1991
II.	*Bg*	*r-bg*	8	17	Salamini, 1980; Maddaloni et al., 1989; Hartings et al., 1990.
III.	*Dt*	*r-Dt*	8	14	Rhoades, 1938; Brown et al., 1989
IV.	*En*	*I*	3	13	Peterson, 1953, 1960; Periera et al.,1985
	Spm	*d-Spm*	3	13	McClintock, 1954; Masson et al., 1987
	Mpl-1	--	3	13	Weydemann et al., 1988
V.	*Fcu*	*r-cu*	--	--	Gonella and Peterson, 1977
VI.	*Mrh*	*r-Mrh*	9	80	Rhoades and Dempsey, 1982
					Shepherd et al., 1989
VII.		*Mu1 to Mu7*	9	200 to 500	Robertson, 1978; Bennetzen et al., 1984
	Mu-A2		9	"	Qin et al., 1991
	Mu-R1		9	"	Chomet et al., 1991
	Mu-9		9	"	Hershberger et al., 1991
	Cy	*r-Cy (Mu7)*	9	"	Schnable and Peterson, 1986
					Schnable et al., 1989
VIII	*Mut*	*r-Mut*	--	--	Rhoades and Dempsey, 1982
IX.	Retro elements				
	Bs1	--	6	304-direct	Johns et al., 1985
	Cin1	--	5	6	Shepherd et al., 1984
	Cin2	--	-	-	Blumberg vel Spalve et al., 1990
	Cin3	--	-	-	"
	Cin4	--	-	-	Schwarz-Sommer et al., 1987b
	Stoner	--	-	-	Varagona et al., 1991
X.	*Stowaway*	--	2	11	Bureau and Wessler, 1994
XI.	*Tourist*	--	3	15 to 23	Bureau and Wessler, 1992
XII.	*Tz-86*	--	10	none	Dellaporta et al., 1984b

the receptor elements of these respective elements were analysed molecularly (see Table 2.1 for references). Although the regulatory elements of the *Mutator* system i.e., *Mu-A2*, *Mu-R1* and *Mu-9* were recently cloned not much is known about their regulation (Chomet et al., 1991; Hershberger et al., 1991; Qin et al., 1991).

The *Cin1* elements which belong to the middle repetitive class of DNA (Gupta et al., 1984) possess features of transposable elements (Shepherd et al., 1984) and they are widely dispersed within the maize genome (Blumberg vel Spalve et al., 1990). This suggests that the middle repetitive DNA may probably be derived in part from transposable elements (Freeling, 1984; Nevers et al., 1986). The '*tourist*' transposable element system consists of a large family of elements which are about 133 bp in length. They are present in very high copy number, widely dispersed in many monocotyledonous species and unlike *Cin* elements are frequently associated with coding genes. Based on these features this family is thought to be the maize equivalent of the human *Alu* family (Bureau and Wessler, 1992). Unlike Tourist the Stowaway family of elements are present in both monocotyledonous and dicotyledonous species. They show target site speicficity (TA in 85% of the insertions) and in some cases contain the regulatory sequences of the promoters of the genes in which they are inserted (Bureau and Wessler, 1994).

2.4. The *En/Spm* Transposable Element System of Maize

2.4.1. Discovery of *En/Spm* system

The *En/Spm* system which is one of the well characterized transposable element systems in maize was first discovered by Peter A. Peterson (1953) at the pale green (*pg*)

locus of maize in a population exposed to Bikini Atoll atomic radiation. Barbara McClintock independently discovered the same system and called it *Spm* for *Suppressor-Mutator* system (McClintock, 1954). That these two elements were identical was established genetically almost a decade after their discovery (Peterson, 1965).

2.4.2. Molecular Cloning of *En/Spm* element

The availability of *the Wx* gene as a probe (Schwarz-Sommer et al., 1984) and the genetic isolation of an autonomous *En* element at the *Wx* locus namely *wx-844* allele (Peterson, 1985c) made the molecular cloning of the *En* element possible (Periera et al., 1985). Using the *Wx* probe, a 2.2 kb long non-autonomous *I* element at *wx-m8* (Schwarz-Sommer et al., 1984) and a 8.2 kb autonomous *En* element at the *wx-844* allele (Periera et al., 1985) were isolated. The autonomous *En* at *wx-844* allele is the standard *En* and refered as *En1* (*Spm-s* is similar to *En1*). Sequencing and heteroduplex analysis of these respective elements (i.e., *I* and *En1*) revealed that they were highly homologous and thus confirming the previous notion that non-autonomous elements are deletion derivatives of autonomous elements (Peterson, 1961, 1970b). Sequence analysis also indicated that the *En* element consists of three distinct regions - the terminal inverted repeats (TIRs), the subterminal repetitive regions (SRRs) and the transposase coding regions (Fig. 2.2).

The TIRs which flank the ends of the element, consist of 13 basepair perfect repeats in reverse orientation (Fig.2.2). These TIRs are again flanked by a 3 base pair target site (of the host gene which is duplicated upon element insertion) at either end (Periera et al., 1985). There appears to be no preference for this target site because the various insertions of *En/I* elements in the *A1* gene of maize show the duplication of sites differing in sequence (Masson et al 1987; Schwarz-Sommer et al., 1987a).

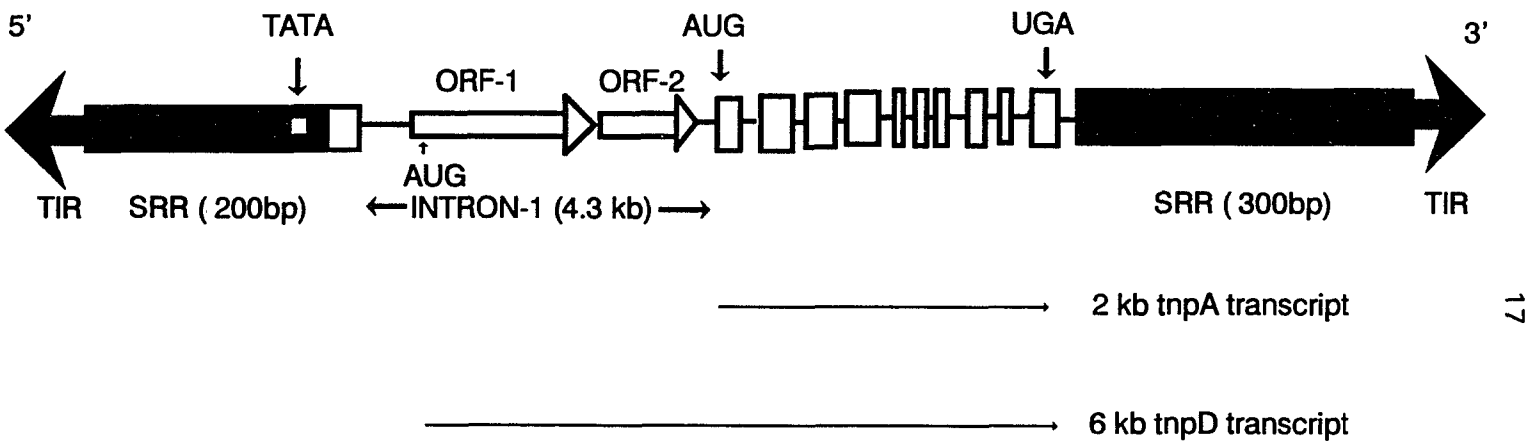


Figure 2.2. Molecular structure of *En/Spm* transposable element.
The large filled arrows are the terminal inverted repeats (TIRs). The shaded boxes represent the subterminal repetitive regions (SRRs). The small open box within the 5' SRR is the promoter. The small arrows within the SRRs are the TNPA binding motifs. The large open boxes represent the exons which encode the transposase A protein. The open arrows within intron-1 are the open reading frames 1 and 2 which encode part of transposase D protein. The tnpA and tnpD transcripts are the result of alternative splicing. (Modified from Gierl et al., 1988).

The SRRs, which span ~200 base pairs on the left end and ~300 nucleotides on the right end of the *En/Spm* element, are composed of short repeats (12bp long) reiterated 9 times on the left end and 15 times on the right end (Fig.2.2). These short repeats are the binding sites for the transposase-A protein and thus act as the cis-determinants of transposition (Schiefelbein et al., 1985; Schwarz-Sommer et al., 1985a; Tacke et al., 1986; Masson et al., 1987; Grant et al., 1990). The subterminal regions along with the TIRs form a 'stem and loop' structure, the formation of which is a prerequisite for element excision (Gierl et al., 1985; Frey et al., 1990; Menssen et al., 1990).

The coding regions of the *En* element consist of 11 exons and 10 introns. The first intron is about 4 kb long and consists of two closely spaced non-overlapping 'open reading frames' (ORF1 and ORF2; Periera et al., 1986). Exon-1 is unusually GC rich (82%) compared to the rest of the *En1* element and contains CG dinucleotides which are targets for methylation (Belanger and Hepburn, 1990; Bird, 1992). The promoter of this transcription unit lies within the left subterminal region with the innermost 12 bp repeat overlapping the TATA box (nucleotide position ~180; Fig. 2.2). The transcription initiation site is located at nucleotide position 209 (Fig. 2.2). The autonomous *En1* encodes four transcripts namely tnpA, tnpB, tnpC and tnpD. The tnpA transcript is 2.4 kb long and the other transcripts vary from 5 to 6 kb in length (Periera et al., 1986; Masson et al., 1989). Evidence for the notion that these different types of transcripts are produced by alternative splicing came from three sources. First, the cDNA sequence of tnpA indicates that it includes all the 11 exon sequences. Second, the larger transcripts include sequences from all the exons plus ORF1 and ORF2 sequences present in the first intron. Third, the

existence of two intron donor splice sites 5' of the ORF1 in the first intron of *En* (Periera et al., 1986; Masson et al., 1989; Frey et al., 1990).

In maize the tnpA transcript is about 100 fold more abundant than the other transcripts (Periera et al., 1986). The tnpA transcript encodes for a 63 kd protein called transposase A (TNPA) and the larger tnpD transcript (6 kb) encodes a 132 kd protein called transposase D (TNPD). Molecular and biochemical analyses showed that the TNPA protein, which possesses both DNA binding and dimerization domains, specifically binds to the 12 bp repeats present in the subterminal region (Gierl et al., 1988a; Grant et al., 1990; Trentman et al., 1993). However, such information is not yet available for the TNPD protein. It was shown that only the products of tnpA and tnpD transcripts are adequate for *En* transposition (Frey et al., 1990; Masson et al., 1991). The functions of tnpB and tnpC transcripts are not well understood.

2.4.3. Genetic functions of *En/Spm* and their molecular basis

Genetic analyses have revealed that the autonomous *En/Spm* element interacts differently with diverse non-autonomous *I* elements. This variable interaction depends on the expression of *En/Spm* which manifests itself in the form of four main genetic functions namely the suppressor (S) function, the mutator (M) function, the coexpression (Coex) function and the activator (A) function (McClintock, 1965b; Nevers and Saedler, 1977; Peterson, 1988). However, molecular analysis has revealed that only two element encoded products, transposase A and transposase D, are responsible for three of those functions (Frey et al., 1990; Masson et al., 1991; Banks et al, 1988, Banks and Fedoroff, 1989; Schläppi et al., 1993).

2.4.3.1. S function The S function is discernible only with certain suppressible alleles such as *a-m1 5719 A, a-m1 1112, a2-m1*(class II state), *c2-m2* etc., which are not inactivated completely by the *I* element insertion (McClintock, 1952, 1955, 1957, 1964, 1967; Tacke et al., 1986; Peterson, 1987, 1988). The expression of these alleles, which are termed suppressible alleles (Masson et al., 1987), varies from pale to wild type color in the absence of an active *En/Spm* element. In the presence of an active *En/Spm* this colored phenotype is 'suppressed' resulting in a colorless background. This ability of *En/Spm* to suppress the wild type gene function is termed 'Suppressor function'.

Molecular analysis of suppressible alleles that include *a1-m1* and *a2-m1*(class II state) has revealed that the pale color produced in the absence of *En/Spm* is due to the splicing of the *I* elements from respective RNA transcripts similar to introns (Schwarz-Sommer et al., 1985b; Tacke et al., 1986; Menssen et al., 1990). The suppression of color in the presence of *En/Spm* is due to the inhibition of *A1* (or *A2* as the case may be) transcription by the binding of an element encoded product at the ends of the *I* element present in these genes (Fig. 2.3). This product was later identified as the transposase A whose DNA binding properties and its specific binding to the subterminal 12 bp repeats were clearly demonstrated (Gierl et al., 1988; Grant et al., 1990; Trentman et al., 1993). Among the various subterminal TNPA binding motifs the tail-to-tail motif is proven to be the smallest cis unit that can effectively block transcription in the presence of TNPA (Grant et al., 1990).

2.4.3.2. M Function The ability of the *En/Spm* element to restore the wild type gene action in those alleles which are inactivated by the insertion of the *I* element is termed its 'mutator (M)' function (McClintock, 1965b; Peterson, 1988). Unlike the S

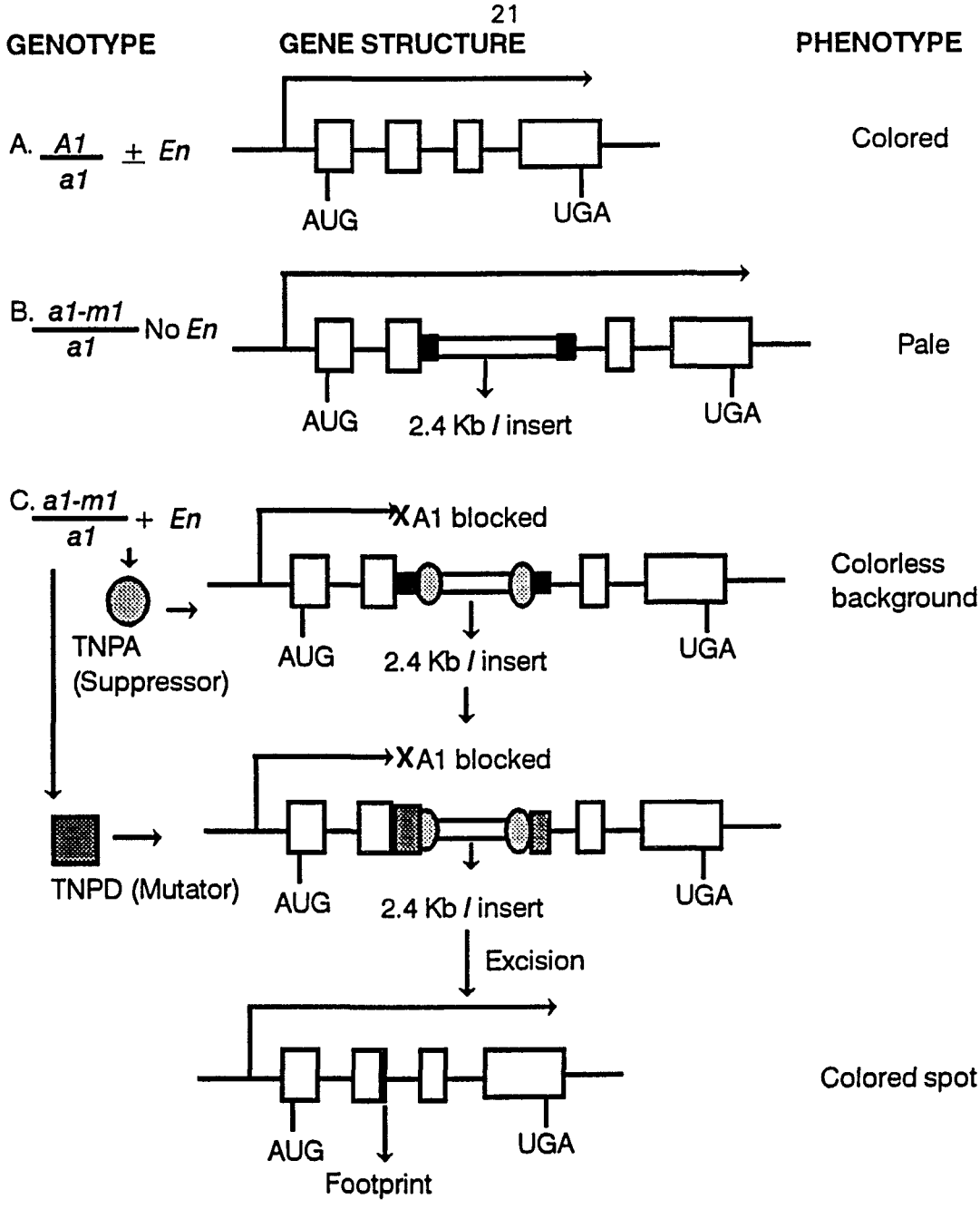


Figure 2.3. Molecular basis of Suppressor and Mutator functions of *En/Spm*. An exmple of the interaction between *a1-m1* and *En1* is shown. The stem-loop structure which is thought to be formed upon TNPA binding to the subterminal regions of the *I* insert is not shown. TNPD binds TIRs. (modified from Schwarz-Sommer et al., 1985a).

function, the M function can be observed with all the known mutable alleles in maize with the exception of *a2-m1* (class II state) allele (McClintock, 1955, 1957, 1958; Menssen et al., 1990). The expression of the M function which is revealed only in the presence of active S function (McClintock, 1965b), can be variable (Peterson, 1970a; McClintock, 1971).

The mutator function of the *En/Spm* element was shown to be encoded by the tnpD transcript. Following the binding of TNPA to the subterminal regions, the TNPD protein binds to the already formed **protein(TNPA)-DNA** complex and excises the *I* element by introducing staggered nicks at the TIRs and thereby restoring the wild type gene function (Frey et al., 1990; Masson et al., 1991; Fig. 2.3). Molecular experiments also showed that TNPD alone can not bring about element excision thus confirming McClintock's prediction that S function (TNPA) is needed for M function (McClintock, 1965b).

2.4.3.3. A function McClintock, while characterizing the interaction between the *I* element at the *a2-m1 (state-II)* allele and *En/Spm* found that an active *En/Spm* element can transactivate the inactive *En/Spm* element (McClintock, 1957, 1958, 1965b, 1971). This transactivating function of *En/Spm* is termed as Activator (A) function (Nevers and Saedler, 1977). Molecularly the TNPA protein of *En/Spm* is also responsible for A-function. Methylation of the CG dinucleotides present around the transcription initiation site and in the first exon leads to inactivation of *En/Spm* elements (Banks et al., 1988, Banks and Fedoroff, 1989). The TNPA protein plays an indirect role in element activation. The binding of TNPA to the 5' subterminal region (where the transcription initiation site

resides) prevents further methylation of newly replicated *En/Spm* elements which results in activation of *En/Spm* functions (Schläppi et al., 1993; Fig. 2.4).

2.4.3.4. The Coex function A reverse genetic phenotype compared to that produced by suppressible alleles (for example *a1-m1*) was observed in certain *a1-m* alleles like *a1-m2 8004* and *a1-m 4412* (McClintock, 1968; Reddy and Peterson, 1985). These alleles are colorless in the absence of *En/Spm* but in the presence of *En/Spm* the phenotype of these alleles is spotted with pale background. This phenotype is the result of the expression of both *A1* gene and *En/Spm* element. The ability of the *En/Spm*

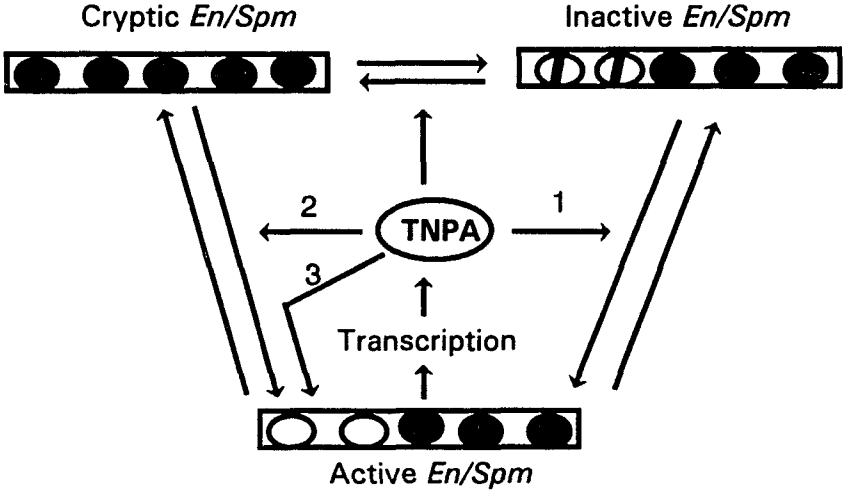


Figure 2.4. Illustration of Activator function of *En/Spm*. The boxes represent the promoter and GC rich downstream control region (DCR). Filled circles represent hypermethylation, partially filled circles represent hemimethylation and open circles represent undermethylation of this region. Binding of TNPA prevents the methylation of the promoter region (the two leftmost circles) and thus transactivating inactive (1) and cryptic (2)elements or maintain its own activity (3; adopted from Fedoroff, 1989b).

element to induce this kind of coexpression is termed as the 'coexpression (Coex) function' (Reddy and Peterson, 1985).

The *I* element insertion in the *a1-m2* allele (which is inserted in the promoter region of *A1* gene) separates the *A1* promoter from its upstream sequences by about 1080 bp (Schwarz-Sommer et al., 1987) thereby inhibiting its transcription which results in colorless phenotype. This observation suggests that the upstream sequences of *A1* promoter probably consists of binding sites for various protein factors that are involved in *A1* transcription. Therefore it was hypothesized that the expression of the *A1* gene in the presence of active *En/Spm* is probably facilitated by some *En/Spm* encoded product (Schwarz-Sommer et al., 1987). An alternative hypothesis was proposed by Masson et al., (1987) who suggested that the colored background observed in the presence of an active *En/Spm* element is due to transcription of *A1* gene initiated from the promoter present within the left end of the *I* element. This hypothesis also was based on the assumption that an *En/Spm* encoded product is involved in the transcription initiation of *A1* gene. However, which one of the *En/Spm* encoded products is involved in this type of interaction is not clearly shown. The colored spots which are darker than the background color are due to M action of the *En/Spm* element (Masson et al., 1987).

All the above mentioned functions are solely the characteristic of autonomous *En/Spm* elements. Non-autonomous elements do not possess any of these functions. However, certain non-autonomous elements, for example *En-I 102* (Cuypers et al., 1988), possesses the ability to reduce the *En/Spm* functions especially its M function (see sections 2.4.5.1 and 2.4.5.4).

2.4.4. Model for *En/Spm* transposition

A generalised transposition model was proposed by Saedler and Nevers (1985) for transposable elements in plants. There is ample evidence that the same mechanism namely "cut and paste" mechanism is also applicable for *En/Spm* transposition in maize as well as in tobacco (Frey et al., 1990; Masson et al., 1991).

According to this model an element encoded transposase binds to the terminii and excises the element by introducing staggered nicks endonucleolytically close to the TIRs. The element-transposase complex remains intact after excision and reinserts itself at the new target site by the same type of endonucleolytic cleavage. The ends of the empty donor site are joined by the plant repair mechanism. During this repair there may be additions or deletions of a few nucleotides which leaves behind a characteristic "footprint". However, Coen et al., (1986) propose that the element excises by precise cutting rather than staggered nicks which occur only during reinsertion of the element at a new target site. According to this model, footprints are the result of processing of hairpin structures, which are formed by joining the two DNA strands at one end, after element excision.

McClintock's initial observation that the mutator function is observed only in the presence of active suppressor function gave a clue that the *En/Spm* element encodes at least two products and both products are involved in element excision (Frey et al., 1990, Masson et al., 1991). The evidence for the requirement of two trans-acting factors for *En/Spm* transposition recently was obtained from transgenic studies where an *I*-GUS construct was introduced into tobacco and its excision (which is revealed as functional GUS) is monitored in the presence of either tnpA or tnpD alone or in combination of both

tnpA and tnpD constructs. These experiments revealed that excision of *I* from the *I*-GUS construct occurs only in the presence of both tnpA and tnpD products (Frey et al., 1990; Masson et al., 1991). The tnpB and tnpC products which are shown not to be required for excision of the *I* element, are thought to function as repressors (Masson et al., 1991) like TNPR (transposase-repressor) encoded by the *En-I 102* element (Cuypers et al., 1988). Taken together these experiments prove that tnpA and tnpD porducts are sufficient to bring about *En/Spm* transposition (Frey et al., 1990; Masson et al., 1991).

Of these two products only TNPA is well characterized biochemically. The TNPA is a novel protein with a DNA binding domain at the N-terminal (located between the aminoacids 122 and 427) and a dimerization domain at the C-terminal (located between the aminoacids 428 and 452). TNPA is unusual in that it does not resemble any of the known DNA binding proteins like zinc finger, leucine zipper etc. However, the composition of this protein resembles that of eukaryotic transcription activators (Lamb and Mcknight, 1991; Trentman et al., 1993). The targets of TNPA are the 12 bp subterminal repeats which are distributed asymmetrically in both terminii. Only six out of nine repeats of the left terminus and eight out of fifteen repeats of the right terminus are involved in TNPA binding (Gierl et al., 1988a). The in vitro DNA binding and immunoprecipitation studies indicated that though TNPA can bind to a single repeat, the binding is high if all the repeats are present. The binding of TNPA to these repeats depends on the composition of the consensus CCGACACTCTTA sequence as indicated by the lack of TNPA binding when the nucleotides at position 3, 10 or 11 of the consensus are altered. The observation that different repeats bind TNPA at different rates also indicates that the

position of the repeats within the subterminal region also is important in binding TNPA (Gierl et al., 1988a).

Recently, the binding of TNPA to monomeric or dimeric (head-to-head or tail-to-tail oriented) 12 bp binding motifs of the subterminal regions was studied using protein-protein and protein-DNA crosslinking experiments (Trentman et al., 1993). Both slow and fast moving complexes are observed with monomeric, head-to-head and tail-to-tail dimers (Fig. 2.5). The fast moving complex is due to binding of one TNPA molecule to the respective 12 bp binding motifs and the slow moving complex is due to binding of two TNPA molecules. However, among the slow moving complexes, the tail-to-tail complex is slightly faster than the other two indicating that the binding of TNPA to tail-to-tail dimer

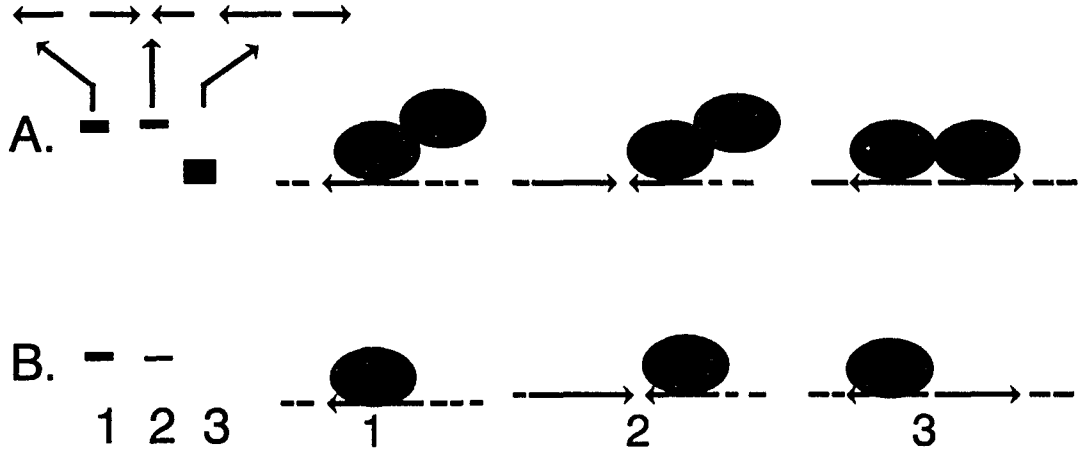


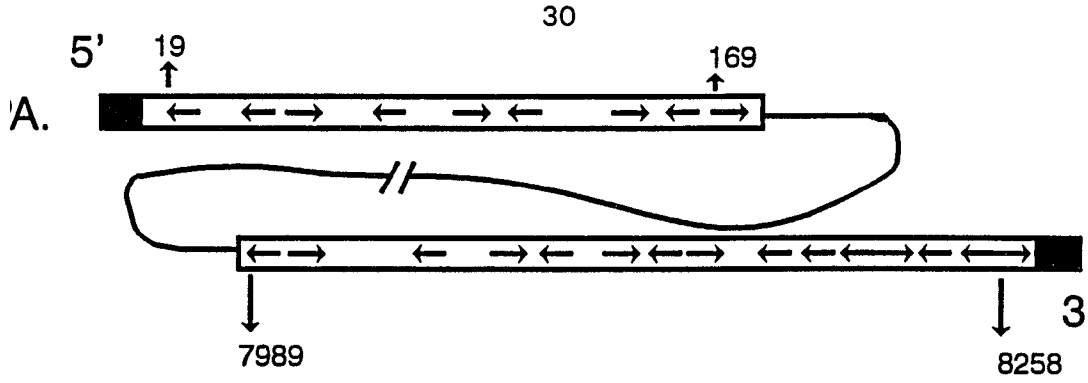
Figure 2.5. Molecular model for TNPA - DNA interaction. The left panel shows gel shift assay and the right panel the model. Lane 1 contains TNPA complexed with monomeric binding motif (arrows). Lanes 2 and 3 contain TNPA bound to either head-to-head or tail-to-tail oriented dimer binding motifs respectively. A) The slow moving band resulting from binding of two molecules of TNPA (shaded ellipses). B) The fast moving band resulting from the binding of one TNPA molecule to the respective motifs. Binding of TNPA to the tail-to-tail motif facilitates the formation of a more compact DNA-protein complex (from Trentman et al., 1993).

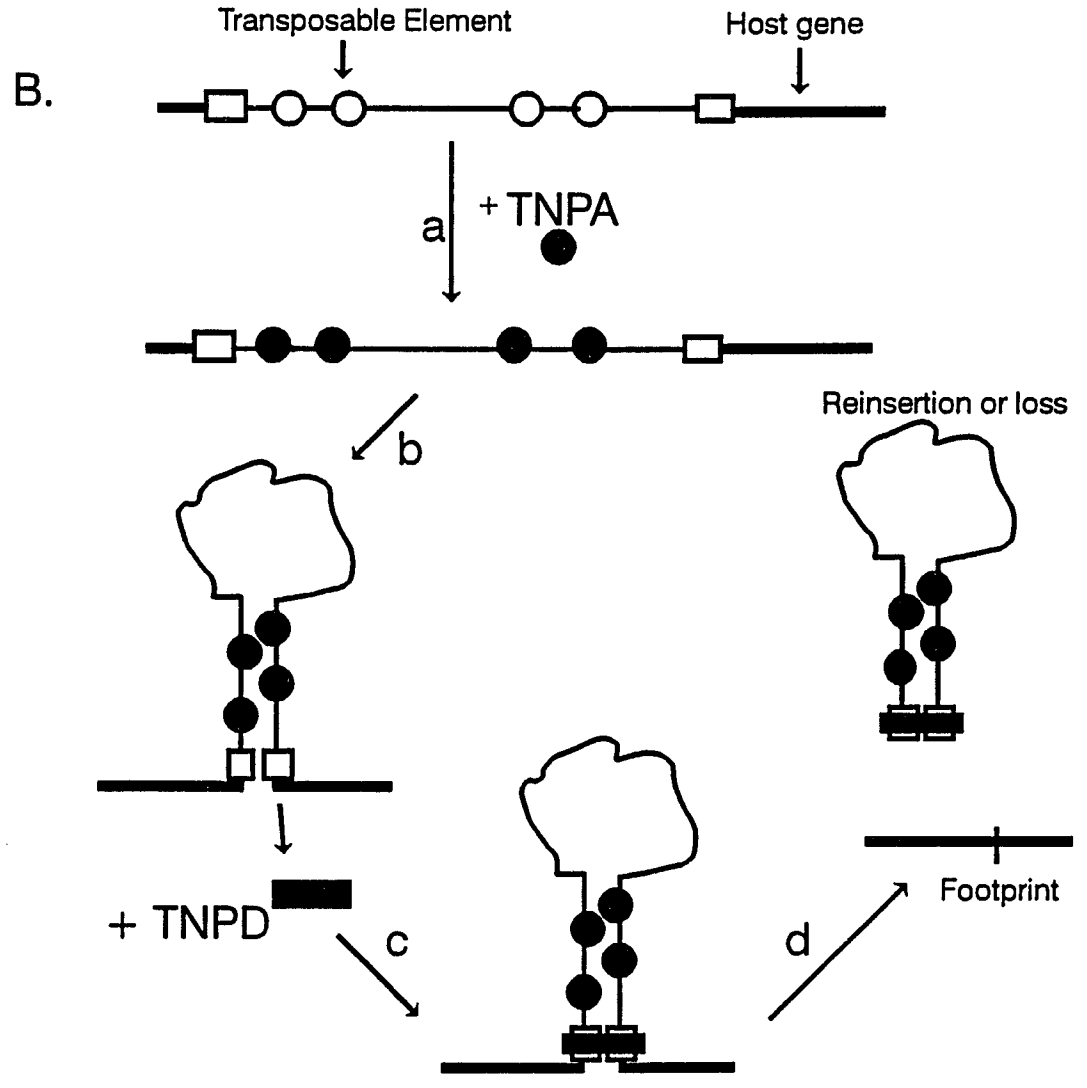
results in the formation of a 'more compact complex' than in the case of head-to-head dimer (Fig. 2.5). The photoactivated DNA-protein crosslinking experiment indicated that two TNPA molecules can be crosslinked to DNA in the case of tail-to-tail complex whereas only one TNPA molecule is crosslinked to DNA in the case of head-to-head complex. This indicates that both TNPA molecules are in direct contact with tail-to-tail oriented binding motifs whereas only one TNPA molecule, due to steric hindrance, is in direct contact with one of the repeats of the head-to-head oriented dimers and the other TNPA molecule is attached to the first TNPA molecule through a dimerization domain (Trentman et al., 1993).

The three main properties of TNPA i.e., the ability to bind to the subterminal binding motifs, the ability to dimerize and the ability to crosslink to DNA (Gierl et al., 1988; Trentman et al., 1993) help the TNPA to serve as a "glue" in complexing with the subterminal regions and thus bringing the ends together (Fig 2.6). The arrangement of the binding sites in the two subterminal regions resembles a zipper i.e., when the ends are brought together, the binding sites of one end fit in the gaps of the other end (Fig. 2.6). The gluey nature of TNPA together with the zipper arrangement helps in the formation of a stable complex between the two subterminal regions of *En/Spm*. Evidence for the requirement of a complex formation between the ends came from the observation that deletion of the binding sites in the subterminal region reduced the frequency of excision (Schwarz-Sommer et al., 1985a; Tacke et al., 1986; Masson et al., 1987; Schiefelbein et al., 1985; Menssen et al., 1990).

Once the ends are complexed with TNPA the next step will be cleavage of the element ends from the host sequence. This function seems to be carried out by TNPD

Figure 2.6. Molecular model for *En/Spm* transposition.
A. The structured subterminal repetitive regions (SRRs) of *En/Spm* showing the distribution of TNPA binding motifs (small arrows). The numbers indicate the nucleotide position of the first and last motif within each SRR.
B. Diagramatic illustration of the events leading to the excision of an element insertion in a gene (thick line). Binding of TNPA to the motifs (open circles) of SRRs (event a) leads to stem-loop formation (event b) which brings the two TIRs closer together. This stem-loop structure promotes the binding of TNPD to the TIRs (event c) and element excision by the endonucleolytic cleavage near the TIRs by TNPD (event d). The ends of the empty donor site are joined by the host repair enzymes during which a characteristic footprint is added at the site of insertion. The excised element will either be lost or reinsert in another place of the genome. (Modified from Frey et al., 1990).


A.
5'
19
169
7989
8258
3'


B.
Transposable Element
Host gene
a
+ TNPA
b
Reinsertion or loss
+ TNPD
c
d
Footprint

protein (Frey et al., 1990; Masson et al., 1991) which is not well characterized biochemically. Evidence for TNPD's role, which is rather indirect, comes from the observation that mutations in tnpD, for example as in *En-2* or *Spm-w,* reduced or abolished excision (McClintock, 1957; Masson et al., 1987; Gierl et al., 1988b; Dash, 1991; Fig. 2.7). The similarity of tnpD with that of members of the CACTA super family of transposable elements, which includes *Tam-1* from *Antirrhinum* (Sommer et al., 1988), *Tgm* from soybean (Rhodes and Vodkin, 1988) besides *En/Spm*, also supports the observation that TNPD will probably bring about excision by interacting with the 13 bp TIRs (Gierl et al., 1989; Frey et al., 1990). The role of TIRs, which along with thesubterminal TNPA binding sites constitute the cis-determinants of *En/Spm* transposition, also is not well understood. The observation that deletion of the two outermost nucleotides of TIRs results in extreme reduction of *En/Spm* excision (Schiefelbein et al., 1988) indicates a possible role for TIRs in *En/Spm* excision (Frey et al., 1990; Masson et al., 1991)

While proof for the generation of staggered nicks close to element ends during *En/Spm* excision and proof for the endonucleolytic properties of TNPD are awaited there has been some evidence supporting the notion that precise double stranded breaks (Coen et al., 1986) rather than staggered cleavage occur during element excision. Using IPCR (inverse PCR) Masson et al., (1991) are able to recover end-joined *En/Spm* terminii indicating the presence of 'linear form of the *Spm* element with blunt ligatable ends' rather than free circular form of *En/Spm* element. Staggered nicks would result in the formation of a free circular form of the element which was observed in the case of *Mu* element transposition (Sundaresan and Freeling, 1987).

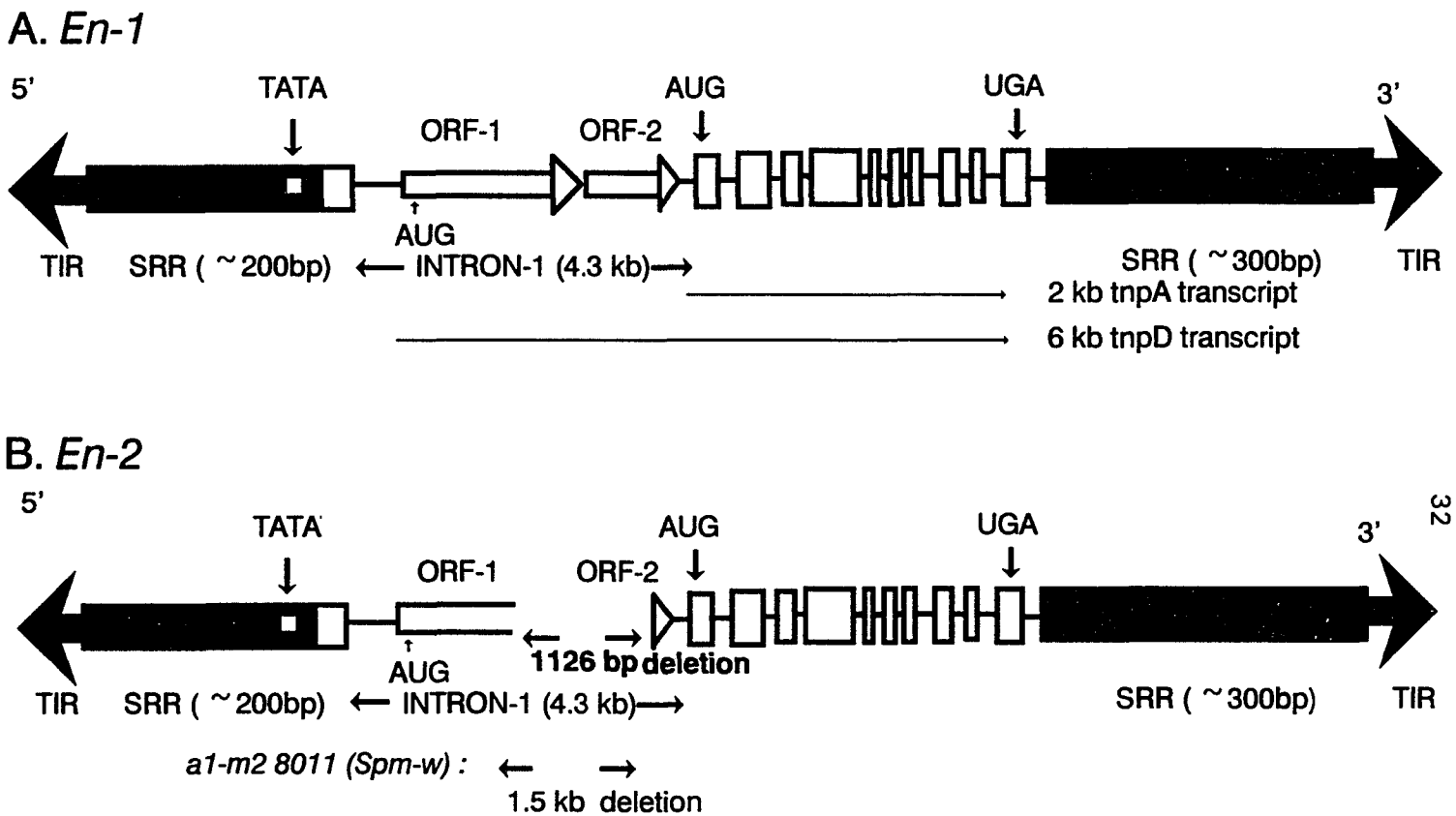


Figure 2.7. Molecular structure of the *En2* element.
Deletion of part of ORF1and ORF2 present in intron-1 of *En1* (A) resulted in the origin of *En2* and *Spm-w* elements (B). The extent of deletion in *En2* and *Spm-w* is shown here. This deletion in ORF1 and ORF2 (which encode TNPD) reduces the M-function drastically.

Both the Saedler and Nevers model (1985) and the Coen et al., model (1986) envisage that reinsertion of the element into a new target site involves generation of staggered nicks at the target site. This concept was suggested based on the observation that the element is flanked by the duplicated target site at its ends (Schwarz-Sommer et al., 1985b). Sequencing of various *En/Spm* and *I/d-Spm* induced alleles in maize revealed that there exists no similarity among the different target sites which indicates that *En/Spm* has no preference for target site (Table 2.2). However, Masson et al (1987) proposes that target site selection depends on the existence of a sequence similar to, if not highly homologous to, the TNPA binding motif in the vicinity of the putative target site. According to their model after the element excision a `TNPA bridge' will be formed between the excised element ends and the TNPA binding motif near the putative target site. This is followed by cleavage of DNA in the vicinity (i.e., at the target site) of TNPA binding site and reinsertion of the element. If this is true it explains the genetic observation that *En/Spm* has a tendency to transpose to closely linked sites (Nowick and Peterson, 1981). For example, if two TNPA binding motif like sequences are available

Table 2.2. Sequence of the target site in some *En/Spm* induced alleles

Allele	Target site	Reference
a1-m1	*TGA*	Schwarz-Sommer et al., 1987
a1-m papu	*ATT*	Schwarz-Sommer et al., 1987
a1-m2	*AAT*	Masson et al., 1987
a2-m1 (states I and II)	*TCG*	Menssen et al., 1991
bt1	*TCT*	Sullivan et al., 1991
bz-m13	*CCG*	Scheifelbein et al., 1988
o2-23	*ACC*	Aukerman and Schmidt, 1993
wx-m8	*GTT*	Schwarz-Sommer et al., 1984
wx-844	*ATA*	Periera et al., 1985

during reinsertion it is probable that the TNPA bridge will be formed more readily using the closer site than the farther site. Taking the various roles played by TNPA into consideration the excess production of tnpA transcript (~ 100 fold greater than tnpD; Periera et al., 1985) would seem logical.

In general the trans-acting factors i.e., TNPA and TNPD provided by the fully functional *En/Spm* element are sufficient to bring about its own transposition and the transposition of other non-autonomous *I* elements. However, a unique type of *I* element recently has been isolated at the *C2* locus which requires in addition to *En*, another independent factor called *mediator* for its transposition (Muszynski et al., 1993). This *I* element named *Irma* for 'Inhibitor that requires mediator also' , is a 3.3 kb element which arose by the insertion of a 1.7 kb non-*En* sequence in the middle of a 1.6 kb *I* element. The subterminal regions of *Irma* are identical with those of *En/Spm* except for a few nucleotide changes in the binding motifs. These nucleotide changes are capable of altering TNPA binding and thus may interfere with the proper zipping of the binding sites of the two ends. *Mediator*, as revealed by the genetic tests, does not possess S or M function and is thus not an *En/Spm* related factor. The product of *Mediator* is thought to provide a 'helper function' for TNPA in stabilizing the element-TNPA complex which is essential for excision (Muszynski et al., 1993).

2.4.5. Regulation of *En/Spm* expression

The *En/Spm* element, which resembles a typical eukaryotic gene, is composed of coding sequences (include both introns and exons) and regulatory sequences i.e., a promoter located within the left end of the element (Periera et al., 1985; Masson et al., 1987; Raina et al., 1993). Like other eukaryotic genes the *En/Spm* element is also subject

to a wide variety of controls both genetic as well as environmental. Because frequent transpositions may increase the mutation frequency of host genes and since most of the mutations are deleterious there is a need to prevent overexpression of the transposable elements (Gierl, 1990). Regulation of element expression is achieved both by element encoded products and host cell regulatory products (Gierl, 1990).

2.4.5.1. Negative regulation of *En/Spm* The *En/Spm* expression is subjected to negative control in several ways (Gierl et al., 1989; Fedoroff, 1989b). The first clues for negative control of *En/Spm* came from McClintock's observation that the active *En/Spm* element can often undergo reversible inactivation (McClintock, 1958, 1971). Later Peterson also identified similar cases where elements are undergoing reversible inactivation in the crown region of the kernels or in the tillers (Peterson, 1966; Fowler and Peterson, 1978).

In general there are three kinds of inactive *En/Spm* elements namely *Spm-i* (*Spm* inactive), *Spm-cr* (*Spm* cryptic) and *Spm-c* (*Spm* cycling). The inactive *En/Spm* elements can be activated spontaneously or by active elements (see Fig. 2.8). The cryptic *En/Spm* elements are only rarely activated by active elements. Whereas the cycling *En/Spm* which was discovered by McClintock while investigating the *a2-m1* (state-II) allele, undergo frequent inactivation and reactivation of activity (McClintock, 1957, 1958, 1959, 1961, 1971; Gierl et al., 1989; Fedoroff, 1989b). Frequently, the *En/Spm* element which is active early in plant development may undergo inactivation in the later stages of plant development indicating that activity of the element is under developmental control (McClintock, 1957, 1971). Such a developmental control has two main components namely 'phase setting' which determines whether the element is active or inactive and

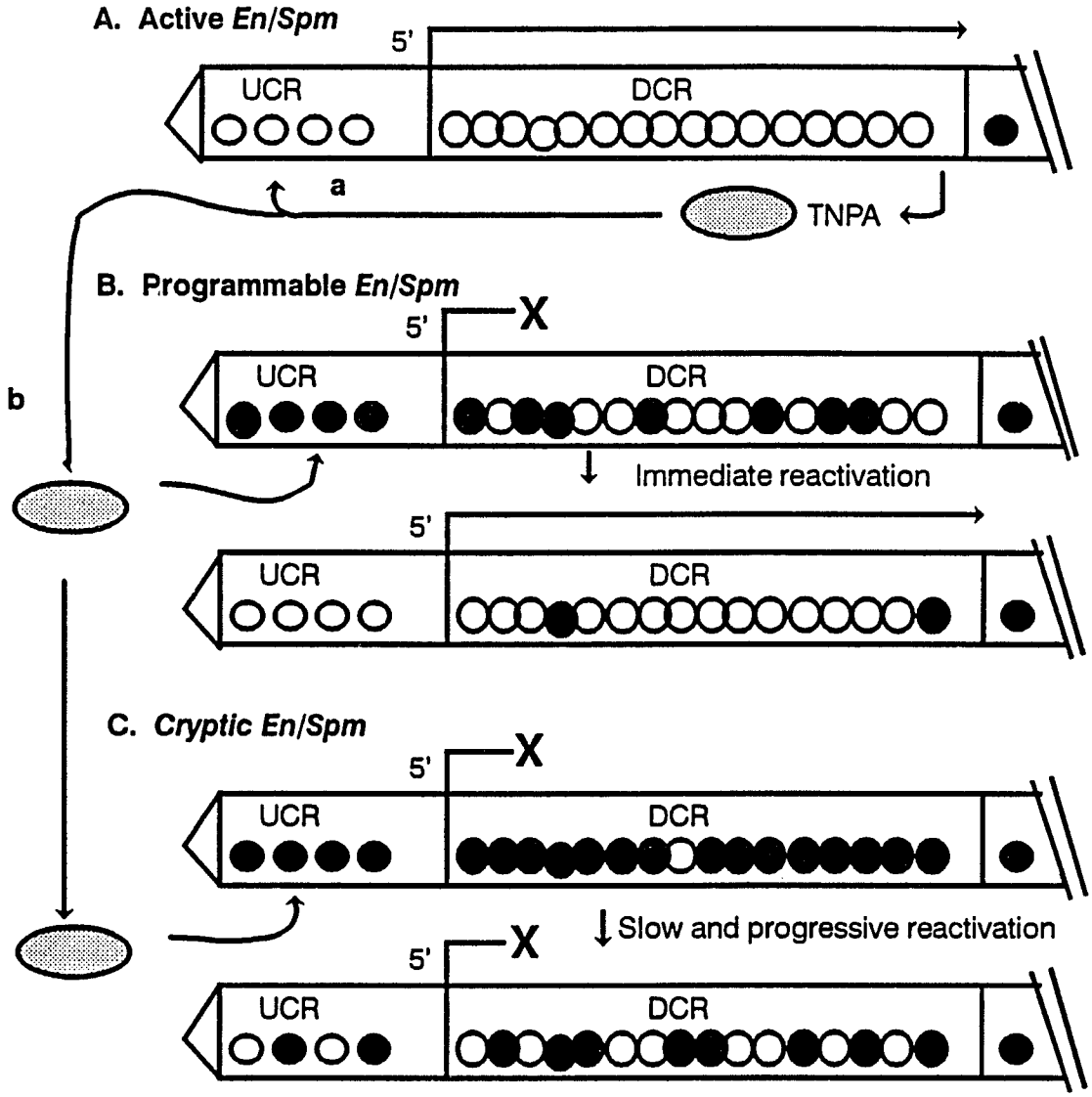


Figure 2.8. Diagramatic illustration of *En/Spm* regulation.
The phenomena of autoregulation (a), positive regulation (b) and negative regulation are illustrated here (see section 2.4.5 for details). UCR is upstream control region (i.e., promoter) and DCR is the downstream control region. The open circles indicate unmethylated C residues and filled circles indicate methylated C residues. TNPA protein (large shaded molecule) is involved in both autoregulation and positive regulation. The programmable *En/Spm* (B) and the cryptic *En/Spm* (C) are transcriptionally silent (denoted by **X**) as a result of negative regulation via methylation. The continuous arrow indicates normal progression of transcription (modified from Banks et al., 1988).

'phase program' which determines the heritability of the phase setting i.e., timing, location and frequency of reversal of activity (Fedoroff and Banks 1988; Fedoroff, 1989a).

The element inactivity is correlated with methylation of C residues in CG and CnG nucleotide sequences present in the upstream control region (UCR; the sequence upstream of the transcription start site) and the downstream control region (DCR; GC rich sequence of the first exon; see Fig. 2.8). None or low levels of C methylation is correlated with element activity whereas methylation of all C residues is correlated with element inactivity. Hypomethylation of C residues only in the UCR and not in the DCR leads to elements activity (phase setting) and hypomethylation of both UCR and DCR leads to heritability of the activation (phase program; Cone et al., 1986; Banks et al., 1988; Banks and Fedoroff, 1989; Fedoroff, 1989a; see Fig. 2.8).

The methylation of *En/Spm* element is under the control of host specific regulatory mechanisms and the element encoded products do not play a role in the methylation of the element. However, some element specific features may also result in low expression of *En/Spm* (Gierl, 1990). One such feature is its unique promoter activity. The *En/Spm* promoter has some special features (Raina et al., 1993).

Sequences required for promoter activity lie within the first 250 bp of the 5' subterminal repititive region (SRR). There are several 9-10 bp sequences of the composition $T_{2\text{-}3}\ A_2\ T_{2\text{-}3}\ A_{2\text{-}3}$ (resembling the TATA box sequence) that are present within the 5' SRR. These sequences also overlap with the 12 bp TNPA binding motifs (nine in number) of this SRR. Deletion of these TNPA motifs reduces the promoter activity and the activity drops by 50 % when the tail-to-tail dimer (which shows strong affinity to the TNPA protein) is deleted (Raina et al., 1993). One interesting finding is that TNPA inhibits

the activity of the unmethylated promoter thus exerting both positive (see section 2.4.5.2) and negative control on element expression (Cook and Fedoroff, 1992; Raina et al., 1993). These observations suggest that the promoter activity depends on multiple sequences present in the upstream control region (UCR; Raina et al., 1993).

The *En/Spm* promoter is insensitive to the enhancer element of the CaMV 35S promoter and this insensitivity is attributed to the lack of optimal TATA box and the presence of GC-rich DCR. By substituting an optimal TATA box sequence and deleting the DCR the promoter can be made highly responsive to the enhancer. The significant implication of this observation is that the DCR might act as a 'silencer' (similar to the one in yeast and rat) and protect the element from the influence of surrounding promoters other than its own (Raina et al., 1993).

The low transcription resulting from the weak promoter activity (Raina et al., 1993) coupled with alternate splicing of the transcripts (Masson et al., 1989) results in very low levels of tnpD (10^{-8} transcripts/cell i.e., less than a transcript/cell) thus making it a rate-limiting component of the *En/Spm* transposition (Gierl, 1990).

The *En-I102* encoded product namely TNPR, for transposase-repressor, may represent another form of element encoded negative regulator (Cuypers et al., 1988). This *En-I102* element reduces the frequency of excision of an autonomous *En1*. The *En-I102* element, which has unaltered promoter and terminii, encodes a 1.8 kb transcript (Fig. 2.9). The TNPR protein, which affects the mutator acivity of *En1* and not its suppressor activity, may exert its negative affect at protein level by acting as a competitive inhibitor of TNPA and/or TNPD in *En/Spm* transposition (Cuypers et al., 1988; Gierl, 1990).

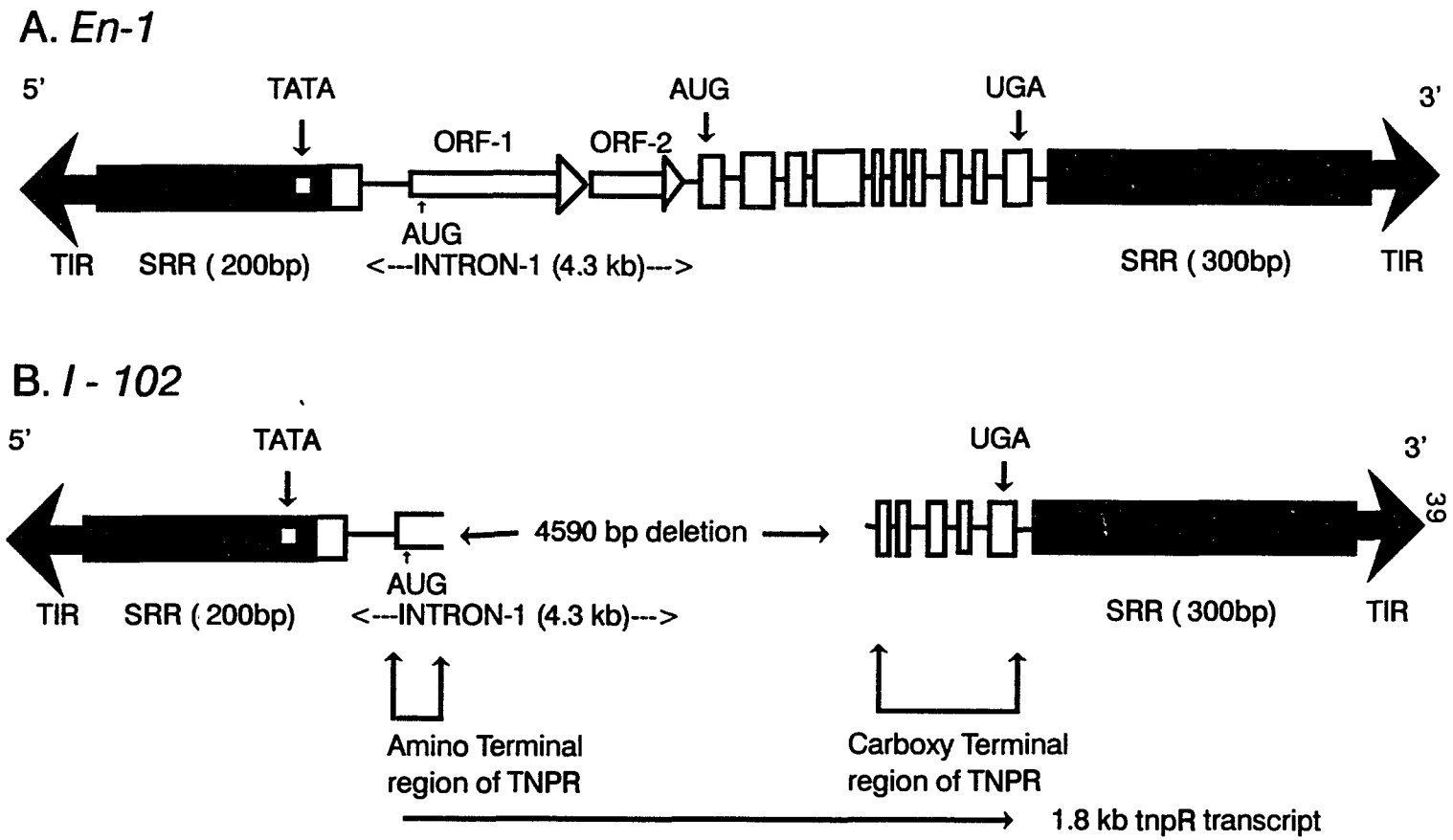


Figure 2.9. Molecular structure of the insert at the *a1-m(r)I102* allele. The *I102* element is a deletion derivative of *En1* (A). The 4.6 kb deletion removes most of the ORFs and four exons. The 3.6 kb *I102* element gives a 1.8 kb transcript which encodes a protein called transposase-Repressor (TNPR). The sequences encoding the amino and carboxy terminal regions of TNPR are marked.

2.4.5.2. Positive regulation of *En/Spm* expression Positive regulation of *En/Spm* involves reactivation of inactive and cryptic elements. The 'inactive *Spm*' elements can get activated spontaneously or by another active element. The 'cryptic *Spm*' elements are activated only rarely by active elements (Fig. 2.8). Whereas the 'cycling *Spm*' undergo frequent inactivation and reactivation of activity (McClintock, 1957, 1958, 1959, 1961, 1971; Gierl et al., 1989; Fedoroff, 1989a). This function of an active *En/Spm* to be able to reactivate inactive elements is termed its activator function (Nevers and Saedler, 1977).

The genetically defined activator function clearly indicated that some element encoded product might be involved in activating the inactive elements. Initially it was hypothesized that TNPA, based on its ability to bind the subterminal motifs, would perform such a task (Masson et al., 1987; Gierl et al., 1988, Fedoroff, 1989a). By binding to the subterminal motifs present upstream of the transcription initiation site, TNPA prevents the methylation of C residues in the UCR which leads to activation of the element. If the C residues in UCR were already methylated it would result in reduction of TNPA binding (Gierl et al., 1988a). However, the undermethylated state of DNA immediately after replication will serve as a better substrate for TNPA binding and thereby avoiding subsequent methylation.

Activation of the element is achieved in a step-wise manner. While inactive elements (which are relatively undermethylated) can be readily activated, the cryptic elements (which are fully methylated) may take several generations before they get activated (see Fig.2.8; Masson et al., 1987; Gierl et al, 1988a, 1989; Fedoroff, 1989a). The role of TNPA in activation was clearly established recently in transgenic tobacco

(Schläppi et al., 1993). In tobacco an inactive *Spm* is activated only in the presence of TNPA and none of the other element encoded products were able to transactivate an inactive *Spm*. The activation is correlated with reduced methylation of C residues in the UCR and maintenance of element's activity requires the continued presence of TNPA (Schläppi et al., 1993).

2.4.5.3. Autoregulation of *En/Spm* The ability of an active element to transactivate an inactive element by preventing methylation of UCR implies that the same mechanism may be involved in the maintenance of its own expression. Thus TNPA may also be involved in the autoregulation of *En/Spm* expression (Gierl et al., 1988a, 1989; Banks et al., 1988; Fedoroff 1989a). The overlapping of the innermost TNPA binding motif of the left subterminal region with the TATA box prevents the overexpression of *En/Spm* (Gierl et al., 1988a). If by any reason the level of TNPA goes down the chances of methylation of UCR would increase and since plants have high methylation maintenance (Gruenbaum et al., 1981) it may lead to inactivation of the element (Gierl et al., 1988a).

2.4.5.4. Modifiers of *En/Spm* expression Quite often many exceptional phenotypes can be observed among transposon induced alleles. Generally those alterations involve either increase or decrease in the number and size of sectors of a particular mutable phenotype, for example, colored spots in the case of kernel variegation. Though these altered phenotypes may immediately suggest that the expression of an autonomous element might have been modified at the transcriptional level, it may not always be the case. A class of independently segregating factors, which are identified by classical genetic means and termed '*modifiers*' may bring about changes in the phenotype not by affecting element expression but by interacting with its products.

Several *modifiers* have been identified in maize that affect the phenotypes of several *En/Spm* induced alleles. The main feature of the *modifiers* is that they affect only the M-function of the autonomous *En/Spm* element. The effect may be either positive (increased spotting) or negative (reduced spotting).

McClintock has characterized a dominant *modifier* which increases the frequency of spotting of a particular *En/Spm* reporter allele by three fold only in the presence of an active *En/Spm* (McClintock, 1956, 1957, 1958). Thus this *modifier* affects only the M-function of *En/Spm* element especially the frequency of mutations. The ability of *modifier* to augment the weak M function of *Spm-w* (*Spm* weak; McClintock, 1957, 1963) also supports the above conclusion. The *modifier* does not have an identifiable phenotype in the absence of an active *En/Spm*. Increase in the dose of this *modifier* does not alter the phenotype proportionally. *Modifier*, like the autonomous *En/Spm* element can also transpose (McClintock, 1956, 1957, 1958, 1965b).

Peterson and his students have identified several modifiers that have a negative effect on the M function of *En1*. The modifier *Restrainer* (*Rst*) alters the coarse spotting phenotype (early excisions resulting in large colored sectors) of *c-m 55292* allele to a fine spotting pattern (late excisions resulting in smaller spots). The *restrainer* does not have M function but whether it shows S-function was not tested (Peterson, 1976b, 1978). Another modifier namely *En-malt*, like *restrainer*, has similar delaying affect on some autonomous *En* induced *c-m* alleles. The *En-malt* also has an M function but was not tested for its S function (Reddy and Peterson, 1983). Still another negatively regulating modifier, the *En-I102*, has no individual phenotype (the colorless *a1-mr* phenotype like

other *I* element insertions). However, it affects the M function (reduced spotting) and not the S function of other *En/Spm* elements (Cuypers et al., 1988; Dash, 1991).

Both McClintock and Peterson believe that these modifiers are deletion derivatives of autonomous *En/Spm* element based on their ability to transpose (McClintock, 1965) and on their ability to show a certain level of M function (Peterson, 1987). The cloning of *En-I102* (Cuypers et al., 1988), which is a deletion derivative of *En1* has certainly proved their point. Certain host specific genes have shown to modify the transposition of P element in *Drosophila* (Rio, 1990). However, such host specific modifying genes are yet to be discovered in maize.

2.4.5.5. Environmental effect on *En/Spm* expression The effect of environmental conditions on *En/Spm* transposition was not well documented. Among various external environmental conditions temperature was shown to affect the transposition rate at *pg-m* locus (Peterson, 1958). Peterson showed that the mutation rate of *pg-m* was higher at 28°C than at 16°C. The mutation rate was higher in younger leaves which have a higher number of actively dividing cells. Based on these observations Peterson concluded that the higher the number of dividing cells the greater the chances for mutations to occur (Peterson, 1958).

Another study addressed the affect of internal environment on transposition. Fowler and Peterson (1978) have characterized a regulatory element namely *En-v* (*En-variable*) which shows reduced expression with the *a2-m(r-papu)* allele in the main stalk. However, the same *En-v* showed increased expression in the tiller of the main stalk. This increased activity was maintained through the next generation eventhough *En-v* was transmitted through the main stalk. Based on these observations they concluded that

some endogenous environmental factors that are present during plant development may cause heritable changes of the regulatory elements.

2.5. States of Transposable Element Function

The mutability of an allele that originated by the insertion of either an autonomous or non-autonomous transposable element is characterized by the timing and frequency of somatic revertant sectors resulting from excision of the transposon from the host gene. Different inserts in a particular gene excise at different times (early or late) and at variable rates (high or low frequency) giving different patterns of a variegated phenotype. McClintock called these variations in phenotype which are characterized by the timing and frequency of excision as **states** (McClintock, 1945, 1946, 1947; reviewed in detail in Peterson, 1987). Both receptor and regulator specific features are involved in the determination of a particular state. These states are heritable (Peterson, 1987) and new states continue to emerge from existing states. McClintock observed that change of state of *I* elements occurs only in the presence of an *En/Spm* element with active S and M functions (McClintock, 1971).

The determination of a state by the *I* element is broadly based on the phenotype conditioned by the *I* insertion in the absence or presence of an active *En/Spm*. In the presence of *En/Spm* the delineation of state is again dependent on the type of response of the *I* element to the S and M functions of *En/Spm* (Schnable, 1986). In the absence of *En/Spm* the phenotype of a particular allele, for example *a1-m1* may vary from very light pale to fully colored (McClintock, 1967).

In the presence of *En/Spm*, the response of *I* elements to S-function may involve **complete suppression** of host gene function (results in colorless phenotype) as in the case of *a1-m1* and *a2-m1* (state-II) alleles (McClintock, 1953, 1958) which are colored in the absence of *En/Spm*. In some cases the host genes are only **partially suppressed** (results in pale background) as for example *a1-m pale(mr)* which is pale in the absence of *En/Spm* (Peterson, 1985a). Occasionally **activation** of the host gene (results in colored phenotype) may also be observed as in the case of *a1-m2* alleles which are colorless in the absence of *En/Spm* (McClintock, 1962).

The response of the *I* element to the M function may involve a total **lack of response** as in the case of *a2-m1* (state-II) allele (McClintock, 1958; Menssen et al., 1990) or **fully responsive**. The number of TNPA binding motifs present in the SRRs and the intactness of TIRs determine the degree of response to M function. The response decreases progressively with the decrease in the number of TNPA binding motifs and deletions within TIRs may completely abolish excision (Schiefelbein et al., 1988; Gierl et al., 1989). The response of the *I* element to the M signal may occur early (resulting in large somatic revertant sectors) or late (resulting in small somatic revertant sectors) in the organ development and at a high (resulting in many spots) or low (few spots) frequency (Schnable, 1986; Peterson, 1987).

The determination of a particular state by an autonomous *En/Spm* will depend on the changes in S and M functions. The timing, rate and tissue specificity of these changes are responsible for different states. In addition *modifiers* can also alter the states produced by an autonomous *En/Spm* with normal S and M functions (Schnable, 1986; Peterson, 1987). The *Spm-w* and *En-2* which are low acting autonomous elements were shown to

have an altered M function (McClintock, ; Dash, 1991) which affect the timing (late occurring) and frequency (few sectors) of excision. The *En* at *a2-m41629* controls a state with late occurring high frequency excisions (Peterson, 1976a). The *En-flow* and *En-crown* elements show tissue specific expression, the former expressing in the basal region of the kernel and the later expressing in the crown region of the kernel (Peterson, 1966).

2.5.2. The basis of states

2.5.2.1. The position hypothesis To explain the changes in the variegation phenotype i.e., differences among states, Peterson proposed that the different states are the resultant of the element being inserted at various positions within the host gene. This hypothesis was based on his observations with newly isolated mutable alleles at *A2* and *C* loci which differ in their states. The *En* element at these new alleles came from a common source whose variegation state does not correlate with that of the new alleles. This implies that the differences in states are due to the new positions occupied by the *En* compared to its donor site (Peterson, 1976a, 1976b, 1977; reviewed in 1987).

2.5.2.2. The composition hypothesis According to the composition hypothesis states are the resultant of quantitative and qualitative differences among the elements that generate those states. This hypothesis was based on McClintock's observation that the *Spm-w* element which is derived from *Spm-s* (standard *Spm*) and characterized by low level of transposition, maintains its state even after transposing to new positions in the genome (McClintock, 1948, 1956, 1957, 1965b).

2.5.2.3. Genetic and molecular evidence Peterson (1981) noted that depending on the situation either model can be evoked to explain the origin of a particular state.

Indeed, there is adequate genetic and molecular data available supporting both of these hypotheses.

The genetic studies of transposed *Mp* from *P-vv* (Khedarnath and Brink, 1958; van Schaik and Brink, 1959), the reconstitution of *P-vv* from different sources of *Mp* (Orton and Brink, 1966), the reconstitution of *Mp-R-Navajo* allele (Brink and Williams, 1973) and the study of newly transposed *Ac2* (Dempsey, 1985) all support the position hypothesis. The various *A1* alleles and the various states derived from original *a1-m2* allele all support the composition hypothesis (McClintock, 1951, 1954, 1955, 1961, 1962, 1963, 1964, 1965a; Reddy and Peterson, 1984).

Molecular data obtained from cloning of different states of *a1-m1* (Schwarz-Sommer et al., 1985a, 1987; Tacke et al., 1986), *a1-m2* (Masson et al., 1987; Schwarz-Sommer et al., 1987), *a2-m1* (state-II) (Menssen et al., 1990) and *bz-m13* derived states (Schiefelbein et al., 1985, 1988; Bunkers et al., 1993) support the composition hypothesis. The *I* elements in these states are deletion derivatives of *En/Spm* and the extent of deletion varies among these *I* elements indicating that quantitaive differences are the reason for differences among states. For example the *a1-m 6078* allele which gives high spotting phenotype in the presence of *En/Spm* gave rise to two low spotting alleles *a1-m1 5719A-1* and *a1-m1 1112*. The number of spots in *a1-m1 1112* are much less compared to *a1-m1 5719A-1*. Molecular analysis indicates that the *I* element in these three alleles are different in the extent of deletion. While the *a-m1 6078* allele has intact terminii with all the TNPA binding motifs, the deletion removes two TNPA binding motifs in the left end of *a1-m15719A-1* and about four TNPA binding motifs in the left end of *a1-m11112*. The stability of the stem-loop structure will be disrupted with a decreasing

number of TNPA binding motifs and thus leads to less frequent excision (Tacke et al., 1986).

Molecular analysis of *wx-m7* and *wx-m9* revealed that the *Ac* elements in these respective alleles are very similar but inserted at a different position within the *Wx* locus. Therefore the differences in the phenotype of *wx-m7* and *wx-m9* are not due to differences in *Ac* but due to their location within the *wx* gene (Muller-Neuman et al., 1984; Pohlman et al., 1984). These findings support the position hypothesis of states. The unstable alleles derived from intragenic transposition of a *Ds* element from *Wx-m5* allele (McClintock, 1953) also supports position hypothesis. In *Wx-m5* (shows wild type phenotype in the absence of *Ac*) the *Ds* element is inserted in the upstream sequences (-450) of *Wx* gene. In the derivative alleles (show null or unstable *wx* phenotype) the element is transposed from -450 to the internal sequences i.e., introns or exons of *Wx* gene (Weil et al., 1992).

Recently Alleman and Kermicle (1993) have isolated 43 unstable alleles of the *R-Sc: 124* gene. Fortytwo of these are due to the insertion of *Ds-6* and one due to the insertion of the *Ds-1* element. The variegation of these alleles ranges from very low to very high kernel spotting. The differences in phenotype among these alleles were attributed to position and orientation of the *Ds-6* element rather than due to change in the composition of *Ds-6* itself. While the *Ds-6* in low spotting alleles is inserted in exons the insert is located in either the introns or in the flanking regions of *R* gene in the high spotting alleles. An interesting finding of this study is that the *Ds-6* element in all these alleles excises at the same rate. This implies that the composition of *Ds-6* remains the same in all these alleles (changes in composition, for example the loss of TNP binding motifs, will usually

result in reduced excision; see section 2.5). Thus the differences in the phenotype of these alleles are clearly due to the position of insertion within the *R-Sc: 124* allele.

Most of the quantitative differences among various *I* elements (resulting from internal deletions of autonomous elements) may also affect the quality of TNPA and TNPD products. Some *I* elements may not encode any product at all but others like *En-I102* encodes a product (TNPR for transposase repressor) that interfere with the normal functioning of TNPA and TNPD. This interference with normal transposase function may delay the action of transposase and thus affect the timing of transposition (Cuypers et al., 1988). The level of transposase may also be affected by other factors like inactivation of autonomous elements which involves methylation of UCR and DCR regions. The reactivation of these elements which involves demethylation at these respective regions may be tissue specific. The specificity of demethylation may in turn determine the tissue-specificity of states (Cuypers et al., 1988).

2.6. Transposable Elements as Introns

2.6.1. General features of introns

There are two main classes of introns namely self-splicing and trans-splicing introns. The self-splicing introns are subdivided into group-I and group-II introns. It is believed that most of the present day nuclear mRNA introns, whose splicing requires the assembly of various proteins which are collectively called 'spliceosome', were derived from the group-II introns (Lambowitz, 1989).

A single general mechanism is thought to be responsible for the splicing of all nuclear mRNA precursors in mammalian, plant and yeast cells. It involves the formation of a lariat structure by the linkage of the 5' terminal G residue (of the 'donor' splice site) to the A residue of the branch site which is 20-50 nucleotides upstream of 3' splice site ('acceptor' site; Sharp, 1987). The splicing system achieves accuracy not by depending on these splice sites but by the presence and appropriate positioning of several different sequence elements to which various SnRNPs (small nuclear ribo-nuclear particles) and proteins must bind in order to form a competent spliceosome, the particle that catalyses the splicing process (Goodall and Filipowicz, 1989).

It has been observed that certain plant introns are efficiently processed in animal systems but the animal introns are not processed by the plant (e.g. dicots) splicing machinery. This finding strongly suggested that the requirements for intron splicing differ between animals and plants especially the dicots (Barta et al., 1986).

2.6.2. Features of introns in plants

In plants more than 80% of the genes contain introns (Goodall et al., 1991). Using synthetic introns it was shown that the minimum functional length of introns in both dicots (e.g. *Nicotiana plumbaginifolia*) and monocots (e.g. *Zea mays*) is about 70 to 73 nucleotides (Goodall and Filipowicz, 1990, 1991). In plants most of the introns studied so far are found to be less than 150 nucleotides in length. The largest intron found in plants is intron-1 of maize transposable element *En/Spm* which is about 4.4 kb in length (Periera et al., 1986). The minimum functional length of plant introns is comparable to that of vertebrate introns but much longer than those in fungi and insects (Goodall et al., 1991).

The sequence comparision of 800 vertebrate, 280 dicot and 146 monocot introns has revealed that monocot and dicot plants have similar GU (5') and AG (3') splice sites like in vertebrates (Brown, 1986; Goodall et al., 1991). However, in two exceptional cases i.e., the intron 1 of soybean *nodulin-24* gene (Katinakis and Verma, 1985) and the newly acquired intron of maize *A2* gene (Menssen et al., 1990) the sequence at 5' splice site is GC instead of GT. Plant introns lack the polypyrimidine tract close to the 3' end which is present in vertebrates. The absence of a polypyrimidine tract, which is involved in spliceosome assembly, does not have any effect on splicing in plants (Goodall and Filipowicz, 1989; Goodall et al., 1991). The putative branch point sequence in plants, like in vertebrates, does not match well with branch point sequences of yeast introns (Brown, 1986; Goodall et al., 1991). Though there is no direct evidence for lariat formation, its formation is assumed based on the observation that mutations in the putative branch point reduces splicing (Goodall and Filipowicz, 1989).

Among plants, the dicot and monocot introns differ significantly from each other in their AT content. While the average AT content of dicot introns is about 74%, it is only 59% for monocot introns (Goodall et al., 1991). The studies with both synthetic as well as natural introns have indicated that in order to be efficiently spliced, introns must be AU rich (at least 68%) in dicots but not in monocots (Goodall and Filipowicz, 1989, 1991). Because of this, monocot as well as vertebrate introns were not efficiently spliced in dicots. However, introns of the maize transposable element *En/Spm* which are relatively AU rich (59-77%), are efficiently spliced when introduced into tobacco and potato (Periera and Saedler, 1989; Masson et al., 1989; Frey et al., 1990). On the other hand the dicot and vertebarate introns are efficiently spliced when introduced into monocots. Eventhough

the AU rich sequences are not necessary for splicing in monocots, reducing the AU content of introns will also reduce the splicing efficiency when the splice sites were suboptimal. However, in the presence of optimal splice sites i.e., GU and AG respectively, reduction of AU content does not have any affect on splicing. Also introns that have secondary structures (stem-loop structures) were poorly spliced in dicots but their splicing was unaffected in monocots (Goodall and Filipowicz, 1991).

These observation indicate that splicing of introns in maize (and in general in monocots) is more **'permissive'** than that of dicots, vertebrates or yeast. The ability of some of the defective transposable elements to be able to splice out from the mRNA transcripts as introns may partly depend on this **'relaxed nature'** of splicing in maize (Goodall and Filipowicz, 1991).

2.6.3. Certain transposable element inserts behave as introns in maize

Transposable element insertions do not always cause gene inactivation. Certain alleles continue to function albeit at various (reduced or normal) levels of wild type gene activity. For example the *a1-m1* allele is pale in the absence of *En/Spm* and the *a2-m1* (state-II) allele is dark purple (McClintock, 1954, 1958). There are several such alleles reported in the literature and in most of them the wild type activity is the result of readthrough transcription and removal of the element from the mRNA transcript (Tacke et al., 1986). However, in few exceptional cases like *wx-B4* and *wx-m1* alleles, the phenotype is null even with readthrough transcription and splicing of the element from mRNA (Varagona and Wessler, 1990; Wessler, 1991).

Although Tacke et al., (1986) first identified the putative splice site consensus sequences in the *I* element inserts of *a1-m1 5719A-1* and *a1-m1 1112* alleles it was

Wessler et al., (1987) who first demonstrated that the *Ds* element of *wx-m9* allele is spliced out from the mRNA transcript like an intron. Since then there were several reports of transposable elements, both retroelements and invert repeat elements, behaving as introns in maize (reviewed in Weil and Wessler, 1990) indicating that splicing from pre mRNA is a common feature of maize transposable elements (Wessler, 1989).

Elements inserted in the antiparallel orientation with reference to the direction of transcription of the host gene are shown to be spliced more efficiently than those inserted in the parallel orientation (Menssen et al., 1990). This is the case with the *a1-m1* allele (Schwarz-Sommer et al., 1985a) and its derivatives (Tacke et al., 1986), *bz-m13* and its derivatives (Kim et al., 1987; Raboy et al., 1989), *a2-m1* (state-I) and its derivative *a2-m1* (state-II) allele (Menssen et al., 1990), *wx-B4* (Varagona and Wessler, 1990), *wx-m9* (Wessler et al., 1987), *adh1-2F11* (Simon and Starlinger, 1987) and *wx-G* (Varagona et al, 1991) alleles. Splicing of the element when inserted in parallel orientation may also be observed but at low levels as in the case of *wx-m8* (Schwarz-Sommer et al., 1984) and *wx-Stonor* (Varagona et al., 1991).

Insertion into either exons or preexisting introns of the host gene does not seem to affect the ability of these elements to function as introns (Weil and Wessler, 1990). However, in a few cases as in *wx-B4* and *wx-m1* (the elements are inserted in exons in these respective alleles) the processed mRNA does not produce functional products thereby giving recessive phenotype (Wessler, 1989).

Though elements as small as 0.4 kb (*Ds1* elements) or as big as 4.3 kb (the *Ds* element of *wx-m9* allele) function equally well as introns (Wessler, 1989), the efficiency of splicing seems to be reduced, in certain cases (eg., *a1-m16078*), with increase in length

of the elements (Tacke et al., 1986). This may be due to the presence of additional signals for splicing and/or polyadenylation in the case of larger elements (Tacke et al., 1986).

The main feature of maize transposable elements that enables them to function as introns is the presence of both donor and acceptor splice sites within the element sequences. The exception being the *Ds2* element in the *adh1-2F11* allele which does not possess any donor or acceptor splice sites but makes use of the donor site of intron-3 of *adh1* gene and a cryptic acceptor site present within the downstream exonic sequence of *adh1* (Simon and Starlinger, 1987). In general the number of donor splice sites present within the element ranges from one to three whereas the number of acceptor splice sites ranges from one to two (Weil and Wessler, 1990). Presence of more donor or acceptor sites as in the case of *Ds1* element at *wx-m1* [3 donor sites and 3 acceptor sites (two in the element and one in the target site duplication); Wessler, 1991] and the *I* elements of *bz-m13* and *a2-m1* (state-I) (2 acceptor sites; Raboy et al., 1989; Menssen et al., 1990) results in alternatively spliced products which may or may not be inframe. But this alternative splicing feature nonetheless increases the chances of producing a functional product (Weil and Wessler, 1990).

The examples of mobile introns (introns created by transposable element insertions) discussed so far still retain their transposon properties i.e., they still transpose actively in the presence of autonomous elements. However, a more stable intron-like element can be evolved from the existing mobile introns. In two such cases , the *bz-m13CS9* derived from *bz-m13* (Raboy et al., 1989) and the *a2-m1* (state-II) allele derived from *a2-m1* (state-I) allele(McClintock, 1956, 1958; Menssen et al., 1990), the *I* elements are more intron-like than their progenitor elements. In each of these alleles the *I* element carries

only a single donor splice site and a single acceptor splice site within the element terminii. The stability of these elements comes from the complete removal of subterminal TNPA binding motifs at one end. While the *I* element at *bz-m13CS9* responds to the M-signal of *En/Spm* at a very low frequency (Raboy et al., 1989), the *I* element at *a2-m1* (state-II) allele does not respond to the M signal but responds to S function of *En/Spm* normally (McClintock, 1958; Menssen et al., 1990). Though these respective elements are better introns than their progenitor elements they are still not 'perfect introns' and probably may continue to evolve into 'true introns'.

2.6.4. Relation to origin of introns

The discovery of introns has led to considerable speculation with regard to their origin. Two prominent theories were put forward to explain the origin of introns. The 'introns-early' hypothesis (Doolittle, 1978) proposes that the progenote, from which the three kingdoms i.e., eubacteria, archebacteria and eukaryotes have evolved, consists of numerous introns and according to this view introns were lost in bacteria but retained in eukaryotes. Gilbert (1978), a supporter of introns-early view, proposes that introns must be present in the progenote because they play a critical role in mediating the recombination of exons. According to this 'exon-shuffling' hypothesis, all the existing protein diversity results from the various combinations of certain number of preexisting exons which are estimated to vary between 1000 to 7000 (Dorit et al., 1990). The 'introns-late' hypothesis (Cavalier-Smith, 1978, 1985) proposes that the progenote contains no or few introns and most of the present day introns are acquired by various lineages in the course of evolution. These two views are mainly concerned with the spliceosome dependent nuclear introns. However, the discovery of various self-splicing introns and their

phylogenetic distribution have led to the view that 'the three major classes of introns are of unequal antiquity' (Cavalier-Smith, 1991). Molecular phylogenetic studies revealed that the self-splicing introns of tRNA (no proteins are involved in splicing) are about 3500 million years old, the spliceosomal introns (both proteins and RNA particles are involved in splicing) are about 1700 to 1000 years old and the third class i.e., the protein-spliced introns (no RNA particles are involved in splicing) probably are intermediate to the above two types (Cavalier-Smith, 1991). More sequence information from hitherto untapped sources like protozoa, archezoa and various groups of bacteria would help in refining these hypotheses (Cavalier-Smith, 1991).

Nuclear introns are thought to have originated by one of several insertion mechanisms such as reverse-self-splicing (of group II introns), transposable elements, duplication of exons followed by activation of cryptic splice sites present within the duplicated segment and finally aberrant splicing may also result in insertion of new introns (Palmer and Logsdon, 1991). Recently several experimental findings indicated that certain group I and group II introns are mobile (reviewed in Lambowitz, 1989; Perlman and Butow, 1989; Cech, 1990). The group II mobile introns, which resemble the non-LTR retrotransposons found in insects, encode a site specific endonuclease (Lambowitz, 1989). These observations led to the speculation that 'the group I and group II mobile introns may represent either introns evolving into transposable elements or transposable elements evolving into introns' (Lambowitz, 1989). However, these mobile introns are different from the present day transposable elements of maize in that their insertions are site specific.

Though the recent reports of maize transposable elements functioning as introns (see section 2.6.3) may serve as an evidence for the transposable-element-origin of certain nuclear introns, examples like this may not serve as sufficient evidence in resolving the issue whether introns are early or late. This is because not all introns are known to resemble transposable elements. Also it is not known whether the most ancient intron known so far i.e., the intron of tRNA-Leu genes of chloroplasts and cyanobacteria (Palmer and Logsdon, 1991) resembles any known transposable elements. Though the discovery of transposable elements in many organisms may lead us to believe that they are ubiquitous, their presence is yet to be revealed in many organisms belonging to various taxonomic groups. The maize examples of transposons behaving as introns though support the 'introns-late' hypothesis, the antiquity of transposable elements itself might have a bearing on this viewpoint.

A recent study on the distribution of *Copia* like retrotransposons (which have long terminal repeats and resemble retroviruses) among various phyla revealed that *Copia* like elements were present in 64 plant species (representing all major lineages of plants) and one protist (Voytas et al., 1992). This finding indicates that the *Copia* like retrotransposons are ancient. Such an exhaustive survey should be undertaken for other kingdoms and for other types of transposable elements (including those of maize) to establish the ancient nature of transposons. If one can show that transposable elements pre-date or coexist with the introns in the early lineages of the progenote then one can hypothesize that transposable elements probably are the primary source of introns. However, in order to support such a claim one should also show that the earliest known intron and the earliest known transposable element resemble each other .

2.7. Stability of Transposable Elements

2.7.1. Identification of stable inserts

Mutability of the alleles which are generated by transposable element insertions is the key diagnostic feature in the identification of transposable elements. However, transposable element insertions do not always cause unstable gene expression. In few cases the elements are stably inserted at a particular gene and never excise from the locus. Thus stable inserts can be defined by the lack of excision ability. It should be noted that the stable insertions resulting from retrotransposition are not considered here because mutability (or instability) is not a characteristic feature of retroelements (eg., *Cin1*, *Cin2* etc., in maize). The retroelement insertions are stably inserted irregardless of whether the respective elements are active or not.

2.7.2. Causes for stability

Genetic and molecular analysis of few stable inserts has revealed that transposable element inserts become stable because of: methylation of the transposable elements (Banks et al., 1988; Banks and Fedoroff, 1989), changes in the elements' cis-determinants for transposition (Schiefelbein et al., 1988; Hehl and Baker, 1989; Menssen et al., 1990; Aukerman and Schmidt, 1993; Bunkers et al., 1993; Healy et al., 1993) and changes in the size of the element itself (Bunkers et al., 1993).

2.7.2.1. Stability related to methylation of transposable elements McClintock observed that active *Spm* transposable elements can undergo inactivation spontaneously (McClintock, 1958,1971). This inactivation is due to methylation of CG and CnG nucleotides present around the transcription initiation site of *Spm* element (Fedoroff et

al,1988) which in turn shuts off the production of 'transposase' the transacting factor needed in transposition. When active autonomous elements get inactivated in this fashion, all the related non-autonomous element inserts at various loci as well as the autonomous element inserts themselves get stabilized. However, this type of stability is transient in that these inactive autonomous elements regain their activity when a different active element is introduced into the system (McClintock,1971). Methylation of the element may also confer stability in a different way. Methylation of C nucleotides present in the subterminal TNPA binding motifs interferes with TNPA binding thereby reducing or totally abolishing their transposition (Gierl et al., 1988a; Schläppi et al., 1993). Eventhough the maize genome consists of 50-100 copies of *En/Spm* related sequences (Periera et al, 1985) not all of them are transposable which may partly be due to methylation of those elements. Recently it has been shown that a cryptic *Ac* element namely *cAc*-11, which does not transpose even in the presence of an active *Ac* element, is hypermethylated (Leu et al, 1992).

2.7.2.2. Stability related to changes in the cis-determinants of transposition

The role of subterminal TNPA binding motifs and TIRs, the cis-determinants in the *En/Spm* transposition, has been described in sections 2.4.2 and 2.4.4. The frequency of excision is reduced proportionally with the reduction in the number of binding motifs. Thus 'stable inserts' may represent one extreme of a spectrum of transposable elements (usually non-autonomous) with a varying number of TNPA binding motifs and excise at varying rates. The stability of a particular insertion results either from the complete loss of binding motifs present at one subterminal region or from the loss of majority, if not all, of the binding

motifs present at both subterminal regions. The following examples of 'stable inserts' provide clear evidence for such a loss of binding motifs.

From the original *Ds* stock (*Ds* proximal to *Wx*) McClintock isolated several *sh* and *bz* mutable alleles (McClintock, 1952,1953). One of them *sh-m 5933* shows a high frequency of chromosome breakage and also a high frequency of reversion. However, the reversion is not always followed by the loss of the resident *Ds* element, which is fairly fixed at its position to the left of *Sh1* locus, and continues to show late occurring low frequency chromosome breakage (McClintock,1952,1953,1954,1955). Molecular analysis of the *sh-m 5933* revealed the presence of a complex 30 kb insert within the *Sh1* gene. This insert is flanked at its 3' end by a double *Ds* strucure, a 2 kb (*Ds-6* like) *Ds* element inserted in reverse orientation in the middle of a different but similar 2 kb *Ds* element, and at its 5' end by a 'one and half *Ds*' structure (deletion derivative of double *Ds*). Apart from the 30 kb insert at the *Sh1* locus, a duplication which includes part of the 30 kb insert (at 5') and the nearby 5' region of the *Sh1* locus itself also is present on the same chromosome. In the revertants, the 30 kb insert at *Sh1* locus is lost but the duplication (which includes 'one and half *Ds*') is retained. In the case of revertant *Sh1-r5* the duplicated *Sh1* region and the 2 kb *Ds* which is part of 'one and half *Ds*' are deleted leaving behind only the 'half *Ds*' which is lacking transposase binding motifs at one end. This 'half *Ds*' is responsible for a continued low frequency of chromosome breakage in the revertant (Döring et al, 1989; Courage-Tebbe et al,1983).

The *En/Spm* induced *a2-m1* (class II state) allele represents another interesting case of stably inserted transposable element. The *I* element at this allele does not excise even in the presence of autonomous *En/Spm* element. This lack of excision ability was

attributed to the complete loss of 5' subterminal binding motifs of the *I* element (Menssen et al,1990).

Similarly, the recently reported *bz-m13* derivative alleles namely CS-13, CS-14, CS-15 and CS-16 [the *I/d-spm* at this allele is similar to the one present in *a2-m1* (class I state) allele which is the progenitor of *a2-m1* (class II state) allele], lost several TNPA binding motifs present in one or both subterminal regions. The insert at CS-16 allele completely lost its 5' subterminal motifs like that of *a2-m1*(class II state) allele. The PCR assay as well as Southern analysis designed to detect somatic excision products of these alleles in the presence of *En/Spm* did not reveal any detectable level of excision. This implies that these inserts are stably inserted in the respective alleles (Nelson and Klien,1984; Bunkers et al, 1993).

The 168 bp *I/d-spm* insertion at the *o2-23* allele is another case of stable insertion. This allele does not show mutability of the opaque gene even in the presence of active *En/Spm* element. The 168 bp *I* element, the smallest *I* element reported so far, has lost all of its 5' TNPA binding motifs and most of this element is composed of binding motifs derived completely from 3' subterminal region. Southern blot analysis did not detect any excision products indicating that the *I*-element at the *o2-23* allele is relatively stable (Aukerman and Schmidt,1993).

Small deletions within the 13 bp TIRs also eliminates element excision completely. A change of state allele derived from *bz-m13* has a complete insert similar to its progenitor but show no excision because the element endured a deletion of a few nucleotides within the 5'TIR (Schiefelbein et al., 1988). The stable *Ac3* element identified in transgenic tomato and the stable *Ac18* element isolated from transgenic tobacco does not transpose

because of deletions in the 5' and 3' TIRs respectively (Hehl and Baker, 1989; Healy et al., 1993).

Thus in most of the cases described above the complete loss of binding motifs at one end abolishes excision of the element. The presence of these binding motifs at both ends is needed in the formation of a stem-loop structure between the subterminal regions which is a prerequisite for transposition (Saedler and Nevers, 1984). Thus in the absence of binding motifs at one end stable stem loop structures can not be formed which in turn abolishes element excision (Menssen et al, 1990).

2.7.2.3. Stability related to size of the insert In the case of CS-13, CS-14, CS-15 and CS-16 alleles derived from *bz-m13*, the size of the inserts are much smaller compared to that of *bz-m13*. The size of the *I* elements at these alleles are 258 bp, 275 bp, 684 bp and 512 bp respectively. With the exception of CS-16 the other three alleles possess at least few binding motifs at both ends and thus have the ability to form a weak stem-loop structure. However, the suppression in these alleles is not complete (only 80 to 90% of that of completely suppressible CS-6 allele) which also is correlated with lack of excision of the respective *I* elements.

Based on these observations it was concluded that apart from the binding motifs, other properties such as secondary structure of the *I* element which might be dependent on the size of the *I* element itself are involved in suppression (Bunkers et al,1993). Even though there is no direct evidence to the above conclusion one can extend the same reasoning to the lack of excision observed in these alleles and the *o2-23* allele. Larger *I* elements might form more stable stem loop structures than smaller *I* elements. In this regard it is interesting to note that the 945 bp long *I* element of *a1-m1112* (Reddy and

Peterson,1984; Tacke et al, 1986), which has an identical number of binding motifs as the 275 bp *I* element of CS-14 allele, excises at a reduced frequency unlike CS-14.

2.7.3. Degree of stability of the transposable element inserts

The footprints generated upon transposable element excisions are thought to provide variation that contributes to the evolution of genes (Schwarz-Sommer et al, 1985b). Also the presence of transposable elements in certain breeding populations led to the idea that some of the genetic variation observed in such breeding lines might be generated from transposable element insertions (Peterson,1987). Thus the extent of stability of transposable element inserts both in the presence or absence of active autonomous elements may indirectly reflect on the degree of stability of the genetic variation created by these insertions.

Neuffer has studied the stability of the *r-dt* and *d-spm* inserts at the *a1* locus in the absence of corresponding autonomous *Dt* and *Spm* elements (Neuffer,1966). These *r-dt* and *d-spm* elements respond normally to their respective autonomous element encoded signals. However, in the absence of *Dt and Spm* respectively these *a1-m* alleles, when exposed to radiation like UV light, X-rays and to chemical mutagens like EMS, did not revert to the wild type *A1* phenotype. No revertant spots (somatic reversion) were observed in 252 x 10^6 chances implying that these respective inserts are relatively stable in the absence of corresponding autonomous elements.

The *I/d-spm* elements of the *a2-m1* (class II state)allele, the stable alleles of *bz-m13* (CS-13,CS-14,CS-15 and CS-16) and *o2-23* allele represent a unique class of non-autonomous elements which did not excise even in the presence of autonomous *En/Spm*. However, these *I* elements still respond to the suppressor signal (TNPA) of *En/Spm*. This

continued interaction of these *I* elements with *En/Spm* element casts some doubt on their stability. In the case of the *a2-m1* (class II state) allele the *I* element insert which acts as an intron (refer section 2.6.3) might have increased the genetic fitness of *A2* gene. Thus studying the stability of such inserts gives us an understanding on the extent of stability of newly acquired introns as well as the newly created genetic variation. However, there is no available data on the stability of these transposable element inserts.

2.8. Pervasiveness of Transposable Element Inserts in Maize

In maize, transposable elements first were uncovered in the materials grown in geneticists' nurseries. The transposable elements *Ac, Spm, Dt, En , Mu, Cy* and *Uq* were identified in genetically unrelated material. For example, *Ac* and *Spm* arose in genetic stocks undergoing BBF cycle (McClintock, 1948, 1854). *Dt* was identified in Black Mexican Sweet material by Rhoades (1936, 1938) and *En* was identified in the genetic material exposed to the radiation of Bikini Atoll atomic test (Peterson, 1953). *Uq* was uncovered in a line treated with BSMV and WSMV viruses (Sprague and McKinney, 1966; Friedeman and Peterson, 1982) and in numerous corn breeding populations (Peterson and Salamini, 1986).

The discovery of various elements led to the curiosity about their distribution in maize stocks other than those present in the geneticists' nurseries. Most of the knowledge about the element distribution among various maize lines came from the work of Peterson's laboratory and their collaborators.

A survey of maize lines from Bolivia, Colombia, Mexico and Venezuela revealed the presence of *En* element in five lines (Gonella and Peterson, 1975). *Dt* and a weak *En*

elements were tracked down in Cuna tribal corn from Colombia along with a new element called *Fcu* (for Factor Cuna; Gonella and Peterson, 1977). Screening of various breeding populations involved in the development of Iowa Stiff Stalk Synthetic (BSSS) and various inbreds subsequently derived from BSSS, for the presence of active transposable elements belonging to different transposable element systems revealed the presence of *Uq* and *Mrh* elements in that material (Peterson and Salamini, 1986). An extension of a similar survey invoving 108 separate entries of maize germplasm revealed the presence of *Uq* element in BSSS, Burrs White, Hays Golden, Iowa Long Ear, Lancaster, Kolumeir, BS11 population, in the inbred I159 and in the reverse protein population of the long-term Illinois Oil and Protein Selection experiment (Cormack et al., 1988). Some of the maize lines from Chile, Italy and Morocco are shown to contain the *Bg* (*Bergamo*) transposable element (Montanelli et al., 1984).

Though the presence of elements belonging to all the known maize transposable element systems is not tested in certain cases, the above mentioned work established that transposable elements are 'pervasive' in a wide variety of maize populations. Based on these observations Cormack et al. (1988) noted that their findings strongly support the notion that transposition activity of these elements might be responsible for the creation of genetic variation observed in the breeding populations (Peterson and Salamini, 1986). The recent finding that the increase in the frequency of *Uq* containing plants in half-sib and S2 progeny recurrent selection progenies and the decrease of the same in the reciprocal recurrent selection also suggest a possible role of transposable elements as agents that create genetic variation (Lamkey et al., 1991). The increase in the *Uq* frequency is thought to be due to "random genetic drift coupled with a selective advantage possibly associated

with a region of the genome linked to *Uq*" (Lamkey et al., 1991). However, the question whether the selective advantage is the result of *Uq* insertion in a particular gene of that segment or is it a mere coincidence that *Uq* happen to hop into that segment without any genetic consequence remains unanswered. Hitherto the observation that transposable elements are capable of altering gene expression both quantitatively and qualitatively (reviewed briefly in section 2.9) is the only experimental evidence to believe in the notion that transposable elements play a significant role in creating genetic variation needed to adapt to the environmental vagaries.

2.9. Consequences of Transposition Events

Genomes of organisms are not as static as initially thought. The organization and integrity of genomes is constantly changing by gene mutations, genetic recombination and chromosomal rearrangements (Griffiths et al., 1993). These changes can occur spontaneously or mediated by a specific genetic mechanism (eg. recombination). Transposable elements which are known to be present in all living organisms may also constitute one such mechanism of altering genome organization. The transposable element induced genetic changes may range from gross chromosomal rearrangements to simple gene mutations. The genetic variation created by these alterations is thought to be important for evolution of the organism.

2.9.1. Chromosomal rearrangements

Transposable elements can create gross chromosomal rearrangements in plants. All four types of classical chromosomal rearrangements i.e., deletions, duplications, inversions and translocations have been shown to be caused by transposable elements.

However molecular evidence for the presence of transposable element generated rearrangements exists only for the first three types. The occurrence of translocations has been demonstrated only cytologically in maize (McClintock, 1950, 1951).

The transposable element induced rearrangements can arise from 'faulty excision' (Döring et al., 1985) which is also refered as 'aberrant transposition'. This faulty excision results when more than two element ends are present in close proximity (four element ends are present in the case of double *Ds* structure which causes chromosome breakage; Döring et al., 1989). If transposase recognises wrong ends and excises them, the resolving of those ends that follows excision may result in rearrangements or chromosome breakage (Döring et al., 1985, English et al., 1993, Weil and Wessler, 1993; also see section 2.7.2.2).

The rearrangements may range from few hundred bases to tens of kilobases (Taylor and Walbot, 1985, Coen et al., 1989 and Fedoroff et al., 1989b). The cytologically observed rearrangements (McClintock, 1950, 1951) and the genetically identified deletions in *Mu* stocks (Robertson and Stinard, 1987; Robertson et al., 1994) may be 100s or 1000s of Kb in length (Starlinger et al., 1985). But such huge rearrangements were not yet identified molecularly.

In maize most of the larger rearrangements are caused by *Ac-Ds* and *Mu* transposable element systems and in *Antirrhinum* mostly by *Tam-3* element (structurally similar to *Ac-Ds* elements). The *En/Spm* element of maize and *Tam-1* of *Antirrhinum* (which is analogous to *En* element) are not known to cause larger chromosomal rearrangements. An *En* element is shown to cause chromosomal breakage similar to *Ac*

(Cormack and Peterson, 1994) but gross rearrangements resulting from chromosome breakage were not yet observed in the case of *En* breaker.

What are the consequences of such gross chromosomal rearrangements ? The two important consequences that are of some significance are : first, rearrangements can bring a certain gene under the control of new regulatory sequences and second, they may alter the gene expression itself directly. In the case of the *bz-m4* allele in maize a short deletion between *Sh* and *Bz* genes brings the *Bz* gene under the control of *Sh1* promoter which results in altered tissue specific expression of *Bz* (McClintock, 1965a; Dooner, 1980; Gerats et al., 1983). Similarly in the case of *niv*^{rec}:531 allele in *Antirrhinum* an inversion resulting from aberrant transposition separates the promoter (by 1 CM) from the coding region of the *niv* gene and brings *niv* under the control of *Cycloidea*^{radialis} gene. This rearrangement not only increases *niv* expression but also alters its expression pattern in various tissues (Lister et al., 1993).

The case of *niv*-525 allele in *Antirrhinum* illustrates another interesting consequence of chromosomal rearrangements. The *niv*-525 allele is 'semidominant' to the wildtype *niv*$^{+}$ allele. The semidominant phenotype is due to an inverted duplication (207 bp) in *niv*-525. The duplication encodes a 40 bp antisense mRNA transcript that binds with normal *niv*$^{+}$ transcript and makes it non-functional (Coen and Carpenter, 1988). This inhibition of niv^{+} allele produces pale flower coloration instead of wildtype dark colored phenotype.

2.9.2. Footprints as sources of genetic variation

Footprints, which are usually less than 10 nucleotides in length, are generated during the processing of duplicated target sequence by the host DNA repair machinery after transposon excision (Schwarz-Sommer et al., 1985b; Peterson 1987; Wessler, 1988;

see also section 2.2.1). During this processing part or all of the duplication is retained which results in the addition of some extra aminoacids in the protein product. These alterations may or may not affect the protein function depending on whether the correct reading frame is maintained or not (Wessler, 1988). The position of these footprints (i.e., the insertion site) within the gene can also affect the quality of the protein product. Thus, depending on the insertion a range of enzymatic activities can be observed in the case of reverted genes (Wessler et al., 1986).

Based on these observations it was proposed that transposable elements can create genetic variation (through footprints) that is important in evolution (Schwarz-Sommer et al., 1985b). Though direct evidence for such a claim is lacking, a survey of intronic sequences (in which sequence changes can be tolerated by a gene and hence have a chance of being maintained and propagated into subsequent generations) of *waxy* gene in two maize lines provided indirect evidence. This survey revealed the presence of several sequence alterations between the wx alleles of these two lines. These alterations are of the type expected from transposon visitations (i.e., footprints; Saedler et al., 1985). The pervasiveness of transposable elements in a wide variety of breeding populations also indirectly supports the evolutionary role of transposons (Peterson, 1987; Gierl et al., 1989).

2.9.3. Transposons as gene tags

The ability of the transposons to move freely within the genome made them ideal tools for tagging and cloning genes of interest. Practically any gene with an identifiable phenotype can be tagged by designing a suitable genetic screen (Shepherd, 1988; Döring, 1989). A gene is said to be tagged when its wild type phenotype is changed to a mutable

phenotype by the insertion of a known transposable element. Such mutable alleles are then tested both genetically and molecularly to confirm the cosegregation of mutable phenotype with the transposable element. Once a gene was tagged the cloned transposable element sequences can be used as a probe to fish out that particular gene (Nevers et al., 1986).

Gene tagging in maize using transposable elements has come a long way since its first use in tagging the *bz1* gene (Fedoroff et al., 1984). Thusfar in maize at least 25 genes have been tagged and cloned using various transposable elements. The most commonly used transposons for tagging are *Ac, En* and *Mu* elements. Generally the frequency of tagging a gene ranges from 10^{-5} to10^{-6} (Döring, 1989). However, the frequency can be increased to 10^{-3} to 10^{-4} if the transposable element is closely linked to the gene of interest (Döring, 1989). Both *Ac* (van Schaik and Brink, 1959) and *En* (Nowick and Peterson, 1981) show a preference to transpose to closely linked sites. This feature has been exploited to construct novel genetic stocks (Chang and Peterson, 1994; see also section 2.2.1.)

Another significant milestone in gene tagging is the successful use of maize transposable elements in tagging genes from other plants (e.g. dicots) especially in those which lack active transposable elements. The *Ac* element has been used to tag *Ph6* gene in *Petunia* (Chuck et al., 1993) and a*lbino (alb3)* gene in *Arabidopsis* (Long e*t* al., 1993) and *En* element has been used to clone a *male sterile* (*MS2*) gene in *Arabidopsis* (Aarts et al., 1993). These early reports of successful gene cloning using maize transposable elements forebode a bright future for their use as popular tagging tools in many of the crop species.

3. MATERIALS AND METHODS

3.1. Maize Kernel

Maize is a monoecious plant with separate male and female inflorescence. The male inflorescence , the tassel, is terminal and the female inflorescence , the ear, is axially located. The ear consists of hundreds of ovaries and each produces a single kernel upon fertilization (Kiesselbach, 1980). Since the kernel phenotype genetically represents the next generation the availability of a large number of kernels following crosses facilitates easy genetic analysis especially for kernel traits (Fedoroff, 1989b).

The maize kernel consists of four distinct regions - outermost, the pericarp; then proceeding inward the aleurone, endosperm and embryo. The pericarp is derived completely from the ovary and resembles the female parent in genetic constitution. Immediately below the pericarp lies the single cell layered aleurone followed by a multilayered endosperm. The aleurone and endosperm are derived from the fertilization of the secondary nucleus (2n) with one of the two sperm (n) and hence are triploid (3n). However, the triploid aleurone and endosperm are genetically identical with the diploid embryo (Kiesselbach, 1980). Many of the kernel specific genes, for example those involved in the synthesis of starch, storage proteins and the color pigments (anthocyanins), have greatly aided the genetic analysis of maize especially that of transposable elements (Peterson, 1987; Fedoroff, 1989b).

3.1.1. Genes expressed in aleurone

All the genes involved in the anthocyanin biosynthesis pathway are expressed in aleurone layer (reviewed in Jayram and Peterson, 1990; Dooner et al., 1991)). Since

mutations in these genes affect color production in the aleurone these genes can be readily identified by screening the kernel phenotype. Some of the important anthocyanin genes are listed in Table 3.1.

3.1.2. Genes expressed in endosperm

The endosperm is the main source of nourishment to the embryo during germination (Kiesselbach, 1980). Thus endosperm is the main storehouse of starch and proteins that are needed for germination. Many genes involved in starch and protein biosynthesis are expressed in the endosperm. Mutations in these genes give a unique and easily identifiable phenotype to the kernel. Some of these mutations are transposable element induced and

Table 3.1. Anthocyanin Pathway genes that express in aleurone

Gene[a] symbol	Name	Chromosome location	Gene product	Mutant phenotype
R	Colored (kernels and plants)	10L	*myc* like regulatory protein	colorless
C1	Colored aleurone	9S	*myb* like regulatory protein	colorless
C2	Colorless	4L	Chalcone Synthase	colorless
A1	Anthocyaninless	3L	Dihydroflavonol reductase	colorless
A2	Anthocyaninless	5S	Dioxygenase (?)[b]	colorless
Bz1	Bronze-1	9S	UDPG-Flavonol 3-O-glucosyl transferase	bronze
Bz2	Bronze-2	1L		bronze

a = Wild type (colored) ; b = Based on its homology to Flavanone 3-hydroxylase gene of *Antirrhinum majus* and *Petunia* (Menssen et al., 1991).
L = Long arm ; S = Short arm

helped not only in understanding the genetic behavior of transposable elements but also in their molecular isolation. Some important genes that are expressed in the endosperm are listed in Table 3.2.

3.2. Genetic Nomenclature

3.2.1. Mutable phenotypes

Transposable elements when inserted into genes shut off gene expression. These insertions are usually highly unstable i.e., during the course of plant development these elements excise from the respective genes which revert back to their normal wildtype phenotype . This phenomenon is called 'reversion'. The elements if excised during gamete formation result in gametes with reverted wild type genes and give rise to

Table 3.2. Genes that express in endosperm

Gene[a] symbol	Name	Chromosome location	Gene product	Mutant phenotype
Bt1	*Brittle-1*	5L	Phospho-oligosaccharide synthase	brittle (shrunken) endosperm
O2	*Opaque-2*	7S	Regulator of b-32 protein	opaque endosperm
Sh1	*Shrunken-1*	9S	Sucrose synthase-1	shrunken kernel
Sh2	*Shrunken-2*	3L	ADPG pyrophosphorylase subunit	shrunken kernel
Su	*Sugary*	4S	Starch debranching enzyme-I	shrunken and sugary kernel
Wx	*Waxy*	9S	NDP-Starch glucosyl transferase	waxy endosperm

a = Wild type [Round kernel, translucent and glossy (non-waxy) endosperm]
L = Long arm ; S = Short arm

progeny with a normal phenotype. Such reversion genotypes are called 'germinal revertants'. In the somatic tissue the excision of the element may not be simultaneous in all the cells or tissue. While the elements may excise in certain somatic tissues they may remain inserted in some other tissues. This variable excision pattern results in a mosaic of tissues where one can see revertant and mutant phenotypes side by side. Such mosaic phenotypes are often called variegated or mutable phenotypes (reviewed in Peterson, 1987). Some working notations of mutable phenotypes that are used in this thesis are given in Table 3.3.

Table 3.3. List of notations and abbreviations used in the text.

A. Some common notations of mutable phenotypes

Notation	Read as	Nature of Mutable Phenotype
C*l* → c*l*	Colored going to colorless	This notation indicates a high mutability pattern. The kernel looks almost colored with few colorless sectors.
c*l* → C*l*	Colorless going to colored	The phenotype shows a clear colorless background with colored spots of variable size and frequency.
Sh → *sh*	Round going to shrunken	Shows small shrunken sectors in an otherwise round kernel.
Wx → *wx*	Non-waxy going to waxy	Represents coarse waxy mutability. Sometimes the kernels almost look non-waxy (very high waxy mutability).
wx → *Wx*	Waxy going to non-waxy	Few non-waxy sectors in mutant waxy background. Sometimes the kernel almost looks waxy (low waxy mutability)

Table 3.3. Continued.

B. List of some commonly used abbreviations

Abbreviation	Explanation
A	Activator function of the *En/Spm* transposable element.
Ac	The transposable element *Activator*.
BFB cycle	Breakage-Fusion-Bridge cycle.
Ds	Dissociation element; deletion derivative of *Ac*.
DCR	Downstream control region; refers to the GC rich sequence of the first exon of *En/Spm* transposable element.
d-Spm	Defective *Spm* element; deletion derivative of *Spm*.
En	Autonomous '*Enhancer*' transposable element.
I	Inhibitor element; deletion derivative of *En* element.
M	Mutator function (TNPD) of the *En/Spm* transposable element.
S	Suppressor function (TNPA) of *En/Spm* transposable element.
Spm	Autonomous '*Suppressor-mutator*' transposable element. This element is analogous to *En* element.
SRR	Subterminal repetitive regions of the *En/Spm* element.
TIR	Terminal inverted repeats.
TNP	Transposase.
TNPA	Transposase A product encoded by the *tnpA* gene of *En/Spm.*
TNPD	Transposase D product encoded by the *tnpD* gene of *En/Spm*.
TNPR	Transposase-repressor encoded by the *En-I102* element.
UCR	Upstream control region i.e., the promoter region present in the 5' SRR of the *En/Spm* element.

3.2.2. Mutable alleles

In maize the gene symbols are usually italicized. Dominant genes are denoted in uppercase letters and recessive genes are denoted in lowercase letters. However, there is no single way of representing mutable alleles. The various ways that are currently in vogue are shown in Table 3.4. In general the mutable alleles are represented by adding the letter **m** (for mutable) to the gene symbol which are usually in lowercase italics since most of the transposable element induced mutations are recessive to wildtype but dominant to the corresponding recessive allele.

3.2.3. Designating patterns of mutability

As described before mutable alleles are characterized by variegated phenotype. Different alleles express different patterns of mutability. The distinguishing features of such mutability patterns are the **timing** and **frequency** of revertant sectors. The timing of reversion may range from early (large sectors) or late (small sectors) in the development. Based on the size of the revertant sectors they are graded from **'a'** (very late excision i.e., sectors limited to single aleurone cells) to **'e'** (very early excision and hence produces a large sector; Reddy and Peterson, 1984). The frequency of reversion can vary from very few to many revertant sectors. The frequency of reversion is indicated by a scale of **1** (one to two revertant sectors) to **10** (many sectors, whereby the kernel looks almost colored in case of kernel color traits; Reddy and Peterson, 1984). Thus using this method fifty patterns of spotting (a to e X 1 to 10) can be identified. For example a pattern 2a indicates that there are four to five single cell spots and a pattern 2a + 3b + 1c indicates that there are four to five single cell spots, seven to ten smaller spots and one to two medium large spots on the kernel.

Table 3.4. The different ways of denoting mutable alleles

Example	Description
1. *a1-m* or *a-m*	Mutable allele generated by the insertion of a transposable element in the *A1* gene. The letter m also is italicized.
2. *a1-m1, a1-m2* etc.	If more than one mutable allele is present at one locus, for example *A1*, they are numbered serially and the number usually follows the letter m and italicized. In this and the following methods one can not identify whether the mutability is an autonomous element induced or non-autonomous element induced unless otherwise stated.
3. *a1-m1 1112*	Sometimes the number of the plant in which a particular mutable allele arose also is included in the identification of mutable alleles. In this case 1112 denotes the plant number.
4. *a1-m(r)*	The letter r (for receptor) is sometimes used to identify non-autonomous nature of the mutability. This method is commonly used for *I/d-Spm* induced alleles.
5. *a1-m(dt), a1-rCy, a1-rMrh*	Three other ways of representing non-autonomous element induced mutable alleles. The letter m or r is followed by the system name in lower or uppercase.
6. *a1-m(Au)*	Autonomous element induced mutable alleles are sometimes represented as in this example (Au = unstable *A*).
7. *wx-844, wx-844::En*	In some cases the autonomous mutable alleles are represented simply by the number or by adding the corresponding autonomous element symbol to indicate the system to which it belongs to.

3.2.4. Genetic crosses

Each cross can be designated either numerically or directly by the genotypes of the plants used in the cross.

3.2.4.1. Numerical representation The genetic nursery is arranged into sub plots called ranges. Each range measures 12' X 200' and consists of 60 rows of plants. The

number of plants within each row ranges from 12 to 15. This arrangement facilitates easy identification of any single plant in the nursery by a set of numbers. An example is given in Figure 3.1.

Numerically a cross can be represented as for example 931605y-1 X 931618. The numbers on the left side of the cross symbol (**X**) represents female parent and the number on the right side represents the male parent of the cross. Sometimes a right slash (/) symbol is used instead of **X** as in 931605y-1 / 931618. In certain cases the cross is also written as 931605y-1 **ON** 931618 in which 931605y-1 is male and 931618 is female. Selfing of a plant is usually represented as the plant number followed by ⊗ symbol as in 931605y-1 ⊗.

3.2.4.2. Genotypic representation In general only the genes whose mode of inheritance is being tested are shown in the genotypic representation of a cross. While writing the genotypes it is customary to indicate homo- or heterozygosity at each locus.

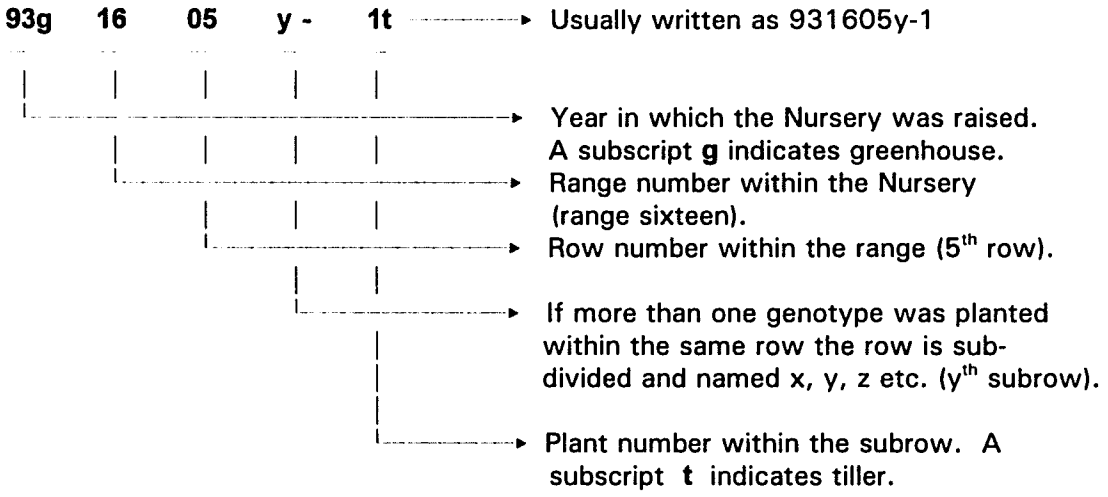


Figure 3.1. Numerical identification of a plant

Since transposable elements usually exist in hemizygous condition only one allele is shown in the genotype. The corresponding allelic position is usually marked by a dash (-). All the linked genes are underlined together and the unlinked genes are usually written in separate groups. Unless otherwise stated all the genes not shown in the genotype are assumed to be homozygous wildtype. The general ways of representing a cross genotypically are shown in Figure 3.2.

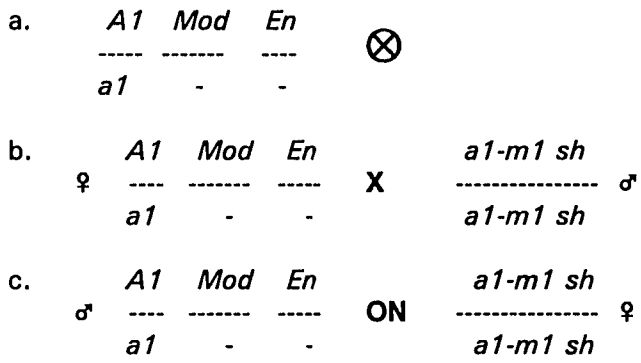


Figure 3.2. Genotypic representation of a cross.
a) Selfing; b and c) Outcrossing (♀ = female; ♂ = male)

3.2.4.3. Phenotypes The phenotype of each parent in the cross is usually written under the respective genotypes. The commonly observed phenotypes are abbreviated for convenience. A summary of phenotype abbreviations used in this work is given in Table 3.5.

Table 3.5. Summary of the abbreviated phenotypes

Abbreviation	Description
cl	Colorless kernel phenotype
Cl	Colored kernels
mot	Mottled kernel phenotype resulting from two doses of ***r*** and one dose of ***R*** allele.
Rd	Plump or round kernels
Sp	Spotted kernels (usually in clear colorless background)
Sp.H	High kernel spotting. This and the following terms are used only in a relative sense and the exact scale of each spotting pattern is indicated in the respective tables in which they were reported.
Sp.L	Low kernel spotting
Cl + Sp	Dark colored spots in pale or wildtype colored background
wx-mC	Coarse (i.e., *Wx* → *wx*) waxy mutability
wx-mL	Low (i.e., *wx* → *Wx*) waxy mutability

3.3. Source Material

The experimental material and the various testers used in this research are listed in Table 3.6. The *a2-m1 wx-844* that is shown in this table and the colored exceptions derived from crossing the above with the *a2 wx* tester are considered as experimental material.

Table 3.6. Description of the experimental materials and testers

Experimental materials / testers	Description
a1 wx or a^0 *wx*	Colorless waxy kernels. Responds to the *Dt* transposable element.
a1 wx::En2	Source of *En2*. The *En2* element is a deletion derivative of *En1* at *wx-844* locus. Shows colorless and low waxy mutable kernel phenotype. In the presence of *En1* it shows coarse waxy mutability.
a1 wx-844	Source of *En1* which is located in intron-8 of *Wx* locus. Shows colorless and waxy mutable kernel phenotype.
a1 sh or a sh	Colorless shrunken kernels. Does not respond to any transposable element.
a1-m(dt)	Reporter allele for *Dt*. Colorless round waxy kernels in the absence of *Dt*. In its presence the kernels are spotted.
a1-m(r)	Reporter allele of *En1*. Kernels are colorless in the absence of *En1* and spotted in its presence.
a1-m1 sh	Reporter allele for *En1*. Colored or dark pale in the absence of *En1* and spotted (b type dark spots and c type pale sectors in colorless background) in the presence of *En1*. Kernels are shrunken in both cases.
a1-m1 Sh wx	Same as above except that the kernels are round and waxy.
a1-m1 sh wx::En2	Low spotted (1-2b) and low waxy mutable. In the presence of *En1* the phenotype changes to high spotted (6-7b + 1c) and coarse waxy mutable.
a1-m1 1112	Kernels are light pale in the absence of *En1*.
a1-m1 1112 En1	Low kernel spotting (1-5a + 1-3b + 1c) in colorless background.
a1-m1 5719A-1	In the absence of *En1* the kernel phenotype ranges from almost colorless to very light pale.

Table3.6. continued

a1-m1 5719A-1 En1	Medium spotted (1-3a+4-5b+1c) in colorless background.
a1-rCy	Reporter allele for *Cy*. Colorless in the absence of *Cy* and spotted in its presence.
a1-rMrh	Reporter allele for *Mrh*. Colorless in the absence of *Mrh* and spotted in its presence.
a1-rUq	Reporter allele for *Uq*. Colorless in the absence of *Uq* and spotted in its presence.
a2 wx	Round colorless waxy kernels. This tester was developed from *a2 bz* (obtained from Ed Coe) and multiple recessive stock (from Maize Genetics Stock Center) testers.
a2 wx-844	Source of autonomous *En1*. Colorless coarse waxy mutable kernels.
a2-m1 (class II state)	This allele [hereinafter called *a2-m1(II)*] is colored (like wild type) in the absence of active *En1* element. In the presence of *En1* the phenotype is colorless sectors in colored background. The intensity of the color of the sectors is the same as seen in the absence of *En1*. Sometimes small colorless sectors can be seen within the colored sectors. This phenotype is variable with different doses of *En1*. One dose of *En1* produces coarse sectored kernel phenotype, two doses of *En1* produce medium sectored phenotype and in the presence of three doses of *En1* the kernels are fine sectored or colorless (Fig. 3.3; McClintock, 1957, 1958). The colorless sectors are due to suppression of *A2* by the binding of TNPA protein to the 3' binding motifs of the *I* element. This *I* element lost most of the binding motifs present at its 5' end and hence can not excise from *A2* gene (see Fig. 3.4). This means that the *a2-m1(II)* alleie can not revert to wildtype *A2*. However, the colored kernel phenotype observed in the absence of *En1* is due to the splicing out of the *I* element from the *A2* transcript like an intron. This splicing of the element from the transcript leaves behind 21 nucleotides which does not alter the reading frame of the *A2* transcript. The addition of seven extra nucleotides does not alter the function of the *A2* protein. The colored sectors observed in the presence of *En* are due to the absence of TNPA protein resulting from the inactivation of *En1* (Menssen et al., 1990).

Table 3.6. continued

a2-m1(III) wx-844	This stock was developed in our lab from *a2-m1(III)* and *wx-844* testers. The kernel phenotype is coarse waxy mutable and fine sectored or colorless. This tester is not homozygous at *wx-844* locus in all plants. Some of the plants are heterozygous i.e., *wx-844 / wx*.
C-I Ds	Reporter allele of *Ac*. This allele is dominant to wildtype *C1* and gives colorless kernel phenotype. In the presence of *Ac* the *C-I* allele will be lost somatically (due to chromosome breakage) and gives sectored kernel phenotype.
r-g	This is a null allele of *R* gene. When homozygous gives colorless kernel phenotype and green plant phenotype. This allele does not respond to any of the known transposable elements.
Line C	This is a color converted W22 line. Contains all dominant anthocyanin genes i.e., *R, A1, A2, C1, C2, Bz1, Bz2* and *Wx* endosperm.

3.4. Kernel Staining

The waxy (*wx*), non-waxy (*Wx*) and waxy mutable (*wx-m*) kernels can be clearly identified when the kernels are colorless. However, when the kernels are colored or highly spotted it is difficult to see the nature of endosperm with regards to *Wx* phenotype. This problem can be obviated by staining the kernels with iodine solution (mixture of I and KI). First the kernels are scraped slightly on a sharpening stone and the diluted I/KI solution (the concentrated I/KI stock should be diluted at lease 10 fold with distilled water before applying to the kernels which prevents dark staining of the kernels) is applied to the exposed endosperm with a paint brush. Within a few seconds the endosperm will be stained and the patterns can be viewed under microscope. The starch granules in *Wx* endosperm are composed of only amylose which stain dark blue. In *wx* endosperm

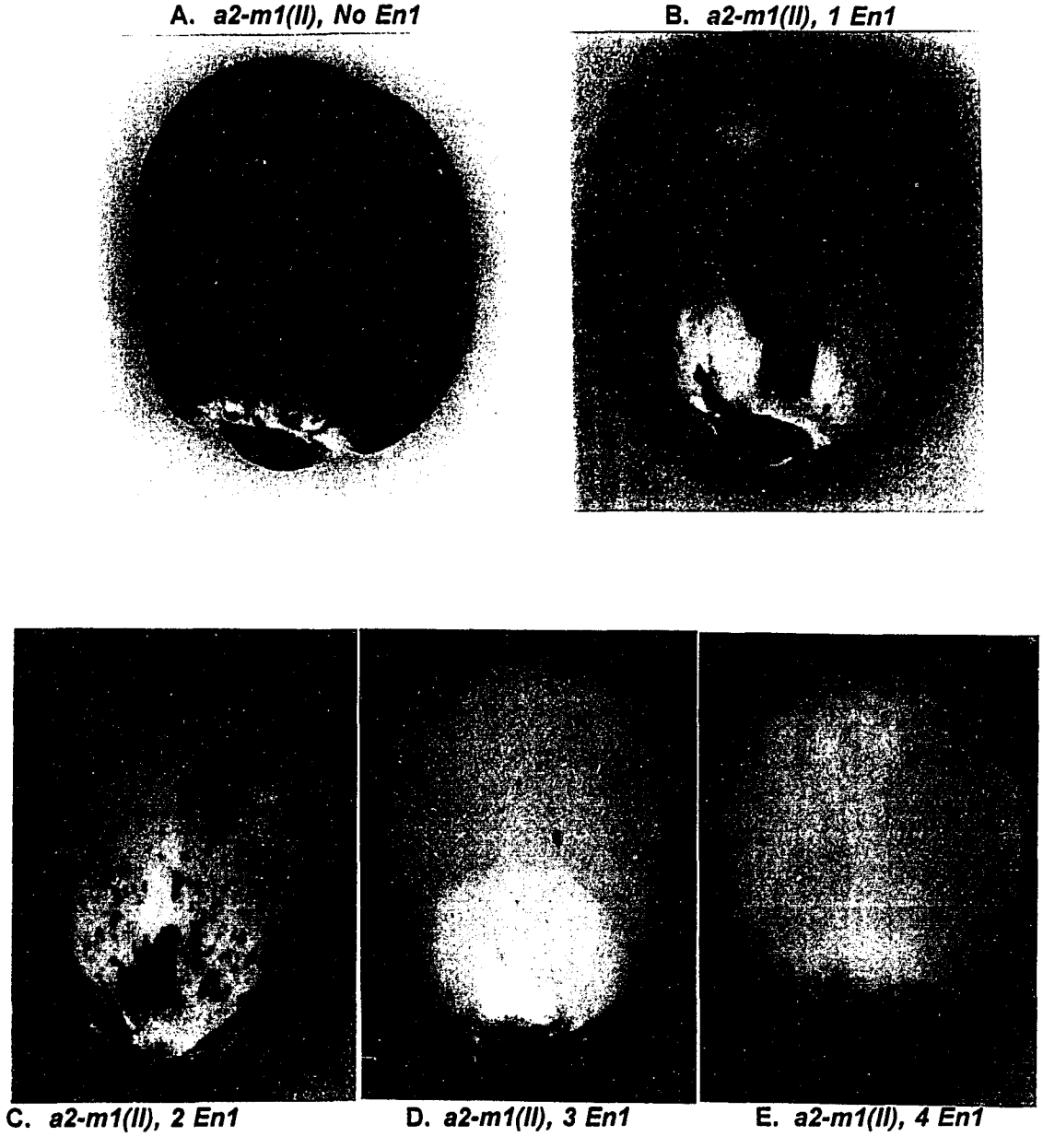
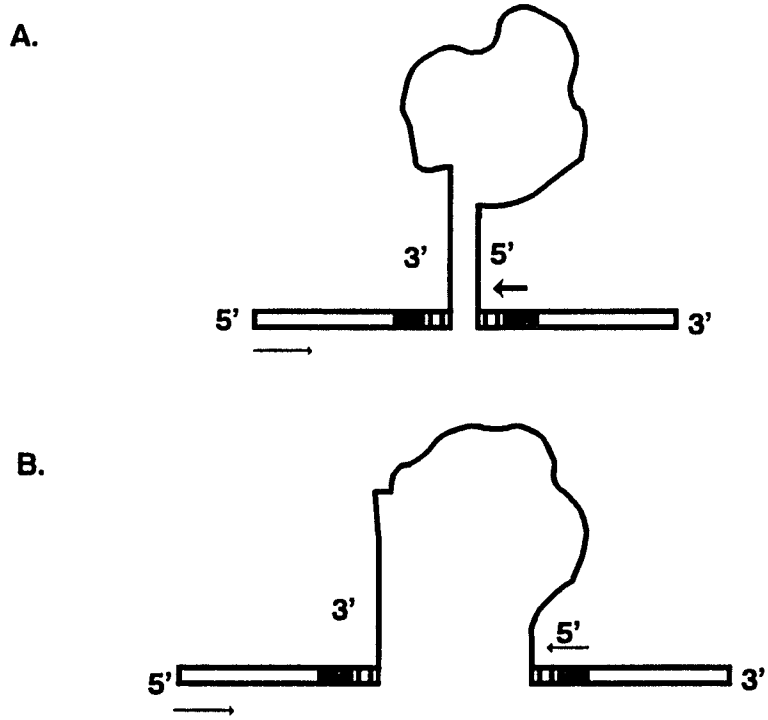


Figure 3.3. Phenotypes of the *a2-m1(II)* allele with different doses of *En1*. The source of *En1* is the *wx-844* allele. Notice the clear waxy mutable phenotype when the kernels are almost colorless (indicated by a small arrow in D and E).



C.

a2-m1(II) DNA	CCTCGCACTACAAGAAAAAAG	GCAAGG.....GACGTTTTCTTGTAG	TGTCGACAGCAGC
Splice consensus	AG	GCAAGG. . . TTT TT TTTT T TG AG R RRRR R	G

Figure 3.4. Molecular configuration of *a2-m1(III)* allele. Open boxes represent 3 bp target site duplication. Hatched boxes represent the 13 bp TIRs. The arrows point to the transcriptional orientation of *A2* and *I/d-Spm* with regard to each other.
A. The 2.2 kb *I* element at the *a2-m1*(class I state) allele, the progenitor of *a2-m1(III)* allele, showing the formation of stable stem loop structure upon binding of TNPA to the subterminal regions which brings the TIRs closer together.
B. The 1.3 kb *I* element at the *a2-m1(III)* allele showing no stem loop structure. Binding of TNPA to the 3' region of the element can not bring the TIRs closer together.
C. Comparison of the 5' and 3' intron splice sites present in the *I* element of *a2-m1(III)* allele with those of plant splice site consensus (modified from Menssen et al., 1990).

amylopectin replaces the amylose sugar and gives brick red stain. The waxy mutability is revealed as sectors of blue and red endosperm upon staining.

3.5. Estimating Number of Transposable Elements

The number of autonomous transposable elements that are segregating in a particular plant can be estimated by crossing that plant to a suitable tester (carrying the non-autonomous reporter allele belonging to the same family as the autonomous element and at a known locus which is usually a kernel specific marker gene). Based on the frequency of kernels with a mutable phenotype the number of autonomous elements can be estimated. For example a plant with the genotype *a1/a1* and containing one autonomous *En* when crossed to homozygous *a-m(r)* tester yields 50% spotted kernels and 50% colorless kernels. The number of spotted kernels varies depending on the genotypes of the parents and the number of autonomous elements. This is illustrated in Figure 3.5. This method of estimation is based on two basic assumptions. First the autonomous element is hemizygous and second the autonomous elements show no dosage affects on the spotted kernel phenotype.

3.6. Molecular Analysis

The two colored exceptions obtained in this study were analysed directly by PCR. Protocols published in laboratory manuals or in journal articles are followed in conducting the molecular experiments.

Genotypes of the Parents	Expected F1 Genotypes	Phenotype	Proportion of each phenotype when ♀ has: 1 *En*	2 *En*	3 *En*
A) *a1* / *a1* X *a-m(r)* / *a-m(r)*	all *a1/ a-m(r)*	Sp (+ *En*)	50%	75%	87.5%
		cl (no *En*)	50%	25%	12.5%
B) *A1* / *a1* X *a-m(r)* / *a-m(r)*	½ *A1/ a-m(r)*	Cl (± *En*)	50%	50%	50%
	½ *a1/ a-m(r)*	Sp (+ *En*)	25%	37.5%	43.75%
		cl (no *En*)	25%	12.5%	6.25%
C) *A1* / *a1* X *a-m(r)* / *a1*	¼ *A1 / a-m(r)*	Cl (± *En*)	50%	50%	50%
	¼ *A1 / a1*				
	¼ a1 / a-m(r)	Sp (+ *En*)	12.5%	18.75%	21.875%
		cl (no *En*)	37.5%	31.25%	28.125%
	¼ a1 / a1	cl (± *En*)			

Figure 3.5. Illustration of the method of estimating the number of autonomous elements

3.6.1. Genomic DNA isolation

Total genomic DNA was extracted from a gram of leaf tissue (collected from ten to fourteen day-old seedlings grown from the colored waxy mutable kernels) using either the Doyle and Doyle (1990) procedure or Dellaporta et al., (1984a) miniprep procedure. The yield of DNA ranged from 25 to 50 μg per gram of leaf tissue.

3.6.2. PCR analysis

An 800 basepair fragment from the DNA of the putative mutant *a2-m1(III)* alleles was amplified using an upstream primer: 5'GCGAATTCATGCATCCATCTCGACGACG3', containing an *EcoRI* site (underlined) and *a2-m1(III)* sequence from nucleotides (nt) 201-

220; and a downstream primer: 5'CGTCTAGATGTGGAGGATGAAGGAGAGG3', which contained nt 1025-1044 and *Xbal* site (underlined). PCR conditions used are the same as described by Weil and Wessler (1993), except that the denaturing temperature was 94°C and samples were cycled 25 times.

3.6.3. Restriction digestion

The PCR reaction mixture was loaded onto 0.8% Agarose gel and run at 80 V for an hour. The 800 bp fragment then was gel purified and digested overnight at 37°C with *EcoRI* and *Xbal* restriction enzymes (from Promega). The vector pGEM32f(+) also was digested with the same enzymes separately (Maniatis et al., 1982; Ausubel et al., 1990).

3.6.4. Cloning of amplified fragment

Standard protocols described in Maniatis et al., (1982) are followed in the ligation of restriction digested fragment and vector, transformation, identification of recombinant clones and in DNA isolation (miniprep) from the positive clones.

3.6.5. Sequencing of the amplified product

The cloned PCR product was sequenced by dideoxy chain termination method (Sanger et al., 1977) using M13 forward and reverse primers.

4. RESULTS

4.1. Genetic Strategy

The main objective of the present study is to identify a change in the state of the *a2-m1(III)* allele (see Table 3.6) which, according to McClintock (1956, 1957, 1958, 1971), is recalcitrant to change in the presence of an active *En/Spm* element. The *a2-m1(III)* allele is the result of an insertion of 1.3 kb *I* element in the intronless *A2* gene. This *I* element was shown to behave like an intron i.e., the *I* element is capable of splicing out from the mRNA transcript of the *A2* gene (Menssen et al., 1990). Since this *I* element behaves like an intron in the intronless *A2* gene and since introns are shown to increase gene expression in plants (Callis et al., 1987), obtaining a change in state of this *I* element (or the lack of a change) will reflect not only on the extent of stability of this *I* element (in the presence of an active *En/Spm* element) but also on the stability of the altered gene expression resulting from the newly acquired introns. It also was shown that this *I* element is stably inserted (never excises) in the *A2* gene (Menssen et al., 1990). Since stable inserts like this (even if they lack intron-like behaviour) are potential sources of genetic variation, the data on the stability of the *I* element of *a2-m1(III)* allele will indirectly reflect on the extent of stability of newly created genetic variation.

In order to monitor changes at *a2-m1(III)* allele a genetic stock homozygous for *a2-m1(III)* and *wx-844* alleles was constructed. The *wx-844* allele is the source of an active autonomous *En/Spm* element (see Table 3.6). This active *En* provides the TNPA and TNPD products which are the two trans-acting factors required for *En/Spm* or *I/d-Spm* transposition (Frey et al., 1990; Masson et al., 1991; see section 2.4.4). McClintock

observed that the change of state of *I* elements occurs only in the presence of an *En/Spm* element with active S (TNPA) and M (TNPD) functions (McClintock, 1971). Therefore we hypothesize that in this stock i.e., *a2-m1(III) wx-844* the continued interaction of TNPA and probably TNPD, with the 3' binding motifs of the *I* element at *a2-m1(III)* allele might generate some mutations in the *I* element and thereby leading to a change of state of the *a2-m1(III)* allele. The possible mutations could be small nucleotide changes in, or deletion of, the remaining 3' TNPA binding motifs or a complete deletion of the *I* element. These changes would eliminate the interaction of *En/Spm* with the *a2-m1(III)* allele and would thereby change the phenotype from colorless (see Table 3.6) to colored kernels even in the presence of an active *En/Spm* element. The screening procedure for identifying such colored kernel phenotypes is explained in the following section.

4.2. Isolation of Colored Exceptions

In order to identify colored kernel exceptions two types of crosses were made. The homozygous *a2-m1(III) wx-844* stock was selfed (Fig. 4.1., cross 1) and outcrossed onto *a2 wx* tester (Fig.4.1., cross 2). All the kernels of the F1 ears obtained from cross 1 (i.e., selfing) carry three doses of *a2-m1(III)* and three doses of *wx-844* (and hence three doses of *En*) alleles. Owing to the high dosage of *En* the expected phenotype of these kernels is fine sectored or almost colorless and coarse waxy mutable (see Fig 3.3). On the other hand all the kernels obtained from cross 2 are *a2 / a2 / a2-m1(III) wx / wx / wx-844* in genotype and hence carry only one dose of *En*. Therefore the expected phenotype of the kernels obtained from cross 2 is coarse sectored and coarse waxy mutable (see Fig 3.3). However, some colored kernels can also be seen segregating in the F1 ears obtained from

cross 1 and cross 2. These colored kernels are separated from the rest and stained with I/KI solution. Based on this staining the colored kernels are classified into non-waxy (stain blue), waxy (stain red) or waxy mutable (blue and red sectors).

The colored non-waxy phenotype is due to reversion of *wx-844* to *Wx* and the excised *En/Spm* is either lost or inactivated. Loss or inactivation of *En/Spm* element leads to absence of TNPA and TNPD products. In their absence the transcription of *a2-m1(II)* allele procedes normally which results in the production of same amount of message as in the case of wildtype *A2* gene (Menssen et al., 1990). The *I* element of the *a2-m1(II)* allele which is transcribed along with the *A2* gene is spliced out from the mRNA transcript leaving behind 21 additional nucleotides in the *A2* transcript. Addition of seven extra

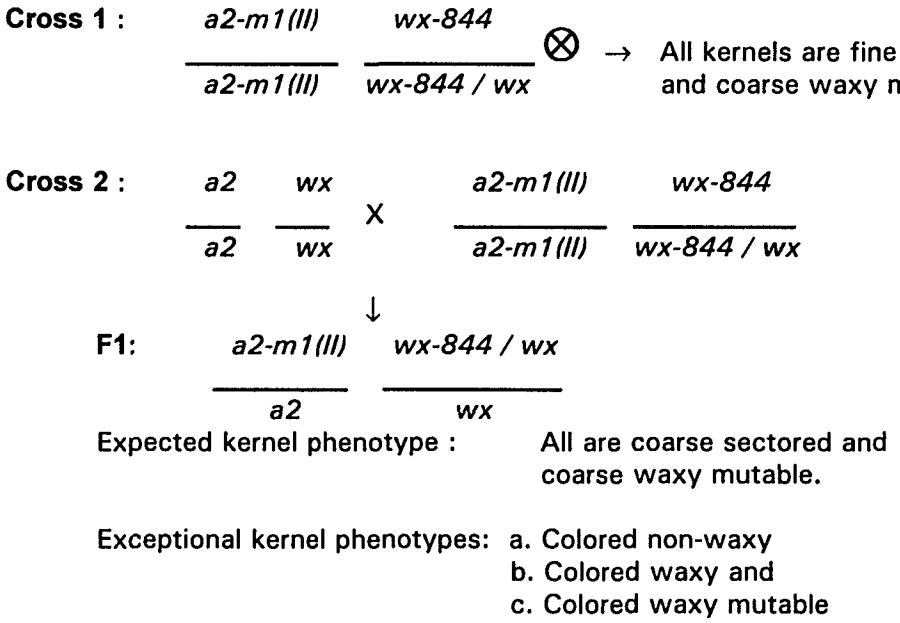


Figure 4.1. Illustration of the genetic strategy employed in monitoring changes in the phenotype of *a2-m1(II)* allele. The exceptions listed in cross 2 are also possible in cross1. The colored waxy mutable phenotype is the unique type.

aminoacids to the *A2* product does not seem to affect its quality because the colored phenotype resulting from this product is indistinguishable from that observed in the case of wildtype *A2* gene (McClintock, 1957).

Similarly the colored waxy phenotype could be due to the loss of *En/Spm* after being excised from the *wx-844* allele which is mutated to waxy. Since the *En/Spm* element is inserted in the intron of *waxy* gene (Periera et al., 1985; Klösgen et al., 1986) the reversion of *wx-844* allele should result in *Wx* phenotype. However, if the excision is aberrant i.e., if the excision also deletes the neighboring exonic sequences it may result in the mutant waxy phenotype instead of the *Wx* phenotype. Alternatively the colored waxy phenotype may be due to inactivation of the *En/Spm* without being excised from *wx-844* allele.

The third type of exception i.e., the colored waxy mutable kernels are the most unique type because they indicate a change in the state of *a2-m1(II)* allele. The waxy mutable phenotype of these exceptions indicates that the *En/Spm* element is active and still present at *wx-844* allele. Since the *En/Spm* element is active in these exceptions, the colored phenotype suggests that the *I* element at *a2-m1(II)* allele is either absent or if present not interacting with the *En/Spm* encoded functions (i.e., TNPA and TNPD). If the *I* element is still present and unmutated the phenotype should be coarse sectored and not colored in the presence of active *En/Spm* element. Thus the lack of interaction between the *I* element and the TNPA product in these colored waxy mutable exceptions might be due to mutations in the *I* element. These mutations could be small nucleotide changes in or deletion of the 3' TNPA binding motifs. Exceptions of this type are analysed by PCR to find out the exact molecular changes that occurred in these exceptions.

The results of the screening are given in Table 4.1. Two colored waxy mutable kernels namely *914038Q and 914039V* were obtained out of 224,651 kernels screened at a frequency of **1 X 10^{-5}**. The high number of colored waxy kernels is rather unexpected considering the insertion of *En* in the intron of *Wx* locus (i.e., in *wx-844* allele).

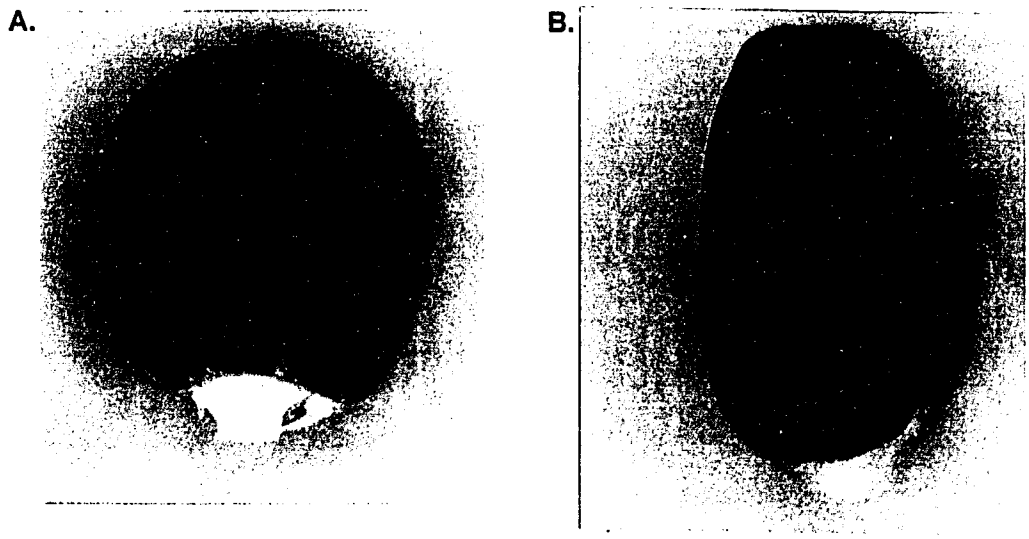
Table 4.1. Results from the crosses (see Fig. 4.1) that were made to isolate changes in state of *a2-m1(II)* allele

	Data from Cross-1	Data from Cross-2	Grand Total
Total number of kernels screened :	54,586	170,065	224,651
Total colored exceptions :	674	7,928	8,602
Colored non-waxy :	231	2,815	3,046
Colored waxy :	443	5,111	5,554
Colored waxy mutable :	0	2	2

4.3. Heritability of the Colored Waxy Mutable Phenotype

Before analysing the two exceptions molecularly the heritability of the colored waxy mutable phenotype of the two exceptions i.e., *914038Q* and *914039V* was genetically tested to confirm that the colored waxy mutable phenotype was not an artifact. The phenotypes of these two exceptions is shown in Figure 4.2. The exception *914038Q* is easily identified by its colored and coarse waxy mutable phenotype. However, the identification of *914039V* exception is quite accidental. The original phenotype of

914038Q



914039V

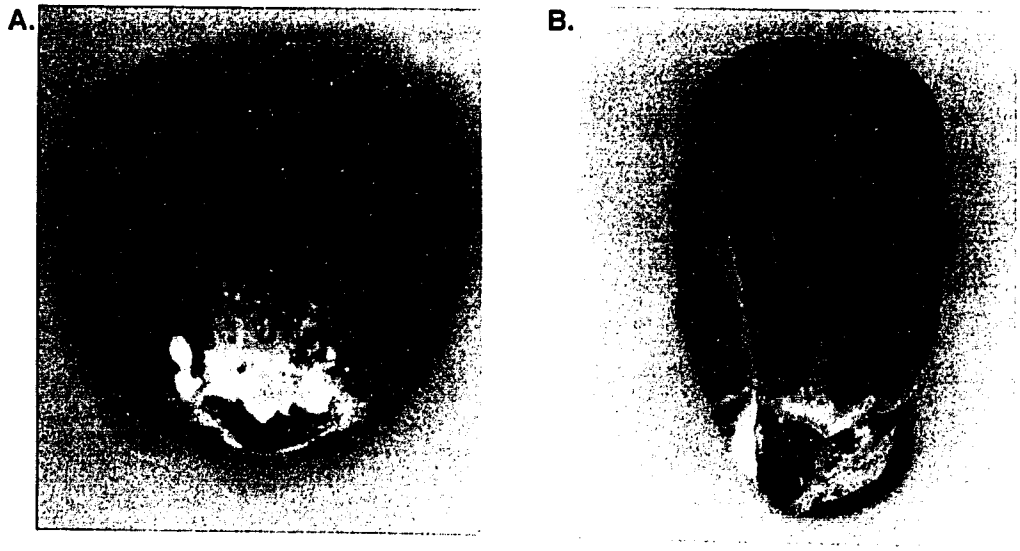


Figure 4.2. Phenotypes of *914038Q* and *914039V*.
A. Colored phenotype; B. Waxy mutable phenotype (indicates the presence of *En1* in these kernels).

914039V is actually colored and non-waxy. This exceptional kernel also showed a tiny colorless sector which was originally thought to be due to late activation of *En*. Thus the exception *914039V* was initially selected to test the heritability of the small colorless sector. These tests showed that the kernels were colored with no colorless sectors. In addition waxy mutable phenotype also reappeared in some of these kernels. Therefore the *Wx* phenotype of *914039V* was thought to be due to early somatic excision rather than germinal excision and hence classified as colored waxy mutable exception. The heritability of the colored waxy mutable phenotype of these two exceptions was tested by selfing and outcrossing the plants grown from these kernels to *a2 wx* tester. The results from these tests are presented in sections 4.3.1 and 4.3.2.

4.3.1. Heritability of *914038Q*

The plant grown from the *914038Q* exception is selfed (cross 1 in Table 4.2) and outcrossed to *a2 wx* tester (cross 2 in Table 4.2). The results from these crosses are shown in table 4.2. The expected phenotypic ratios are 9 colored *wx-m* : 3 colored *wx* : 3 colorless *wx-m* : 1 colorless *wx* in the case of selfed ear and 1 : 1 : 1 : 1 of the same phenotypic classes in the outcross ear. If the two traits i.e., the colored phenotype and the waxy mutable phenotype are considered separately they are expected to segregate as 3 Cl : 1 cl and 3 *wx-m* : 1 *wx* in selfed ear and 1 Cl : 1 cl and 1 *wx-m* : 1 *wx* in the outcross ear. The results are presented in separate sections for colored (section 4.3.1.1) and waxy mutable (section 4.3.1.2) phenotypes of the *914038Q* exception.

4.3.1.1. Colored phenotype The colored phenotype segregated as 81 Cl : 27 bz : 148 cl ratio instead of the expected 3 Cl : 1 cl ratio in the selfed ear (Table 4.2). The colorless kernels are in excess (of ¼ expected) and in addition some bronze kernels,

Table 4.2. Heritability of the colored waxy mutable phenotype of the *914038Q* exception

Cross: 1. *a2-m1(III)** / *a2* 914038Q — *wx-844* / *wx* ⊗ 2. *a2-m1(III)** / *a2* 914038Q — *wx-844* / *wx* ON *a2* / *a2* 914056 — *wx* / *wx*

Colored waxy mutable — Colorless waxy

#	Cross	Colored			bronze [a]			colorless			Total	χ^2 [b]
		Wx	wx-m	wx	Wx	wx-m	wx	Wx	wx-m	wx		
1.	914038Q ⊗	39	82	17	14	30	9	57	113	100	461	NS
2.	914038Q ON 4056	6	44	10	3	6	3	23	122	170	387	NS

* = Putative mutant of *a2-m1(III)* allele ; a = 4 out of 12 bronze kernels in outcross ear are 1-2b spotted ; b = The ratios tested are - Colored : bronze : colorless = 81 : 27 : 148 in ⊗ ear and 9 : 3 : 52 in outcross ear, (*Wx+wx-m*) : *wx* = 3 : 1 in ⊗ ear and 1 : 1 in outcross ear; NS = Nonsignificant

which are not expected, also are segregating in the selfed ear (Table 4.2). Based on these results it can be deduced that the exception *914038Q* is heterozygous for at least four anthocyanin pathway genes. Two of them are *a2* and *bz1* (or *bz2*) and the others may be any two of the remaining three anthocyanin genes i.e., *C1, C2 or A1* (see Table 3.1). Thus, assuming that the exception *914038Q* is heterozygous for four anthocyanin pathway genes, using a branching diagram or the Punnett square a 81 Cl : 27 bz : 148 cl ratio can be derived. In order to find out the source of these recessive alleles the pedigree of the *a2-m1wx-844* and *a2 wx* parents (see cross 1 and cross 2 in Fig. 4.1) that gave rise to the *914038Q* exception is checked. The pedigree check revealed that these recessive alleles must have come from the *a2 wx* parent which is developed from `multiple recessive stock' (which is homozygous recessive at all the anthocyanin pathway loci) and *a2 bz* testers (see Table 3.6). In the outcross ear also the colored phenotype

segregated aberrantly as 9 Cl : 3 bz : 52 cl ratio instead of the expected 1 Cl : 1 cl ratio. This aberrant ratio can be derived if the genotypes of the exception *914038Q* and the *a2 wx* tester are as indicated in Figure 4.3.

The important point that should be noted here is that no sectored kernel phenotype reappeared in either the selfed ear or the outcross ear (see Table 4.2) indicating that the colored phenotype of the exception *914038Q* is indeed heritable. Considering the other recessive genes contributed by the *a2 wx* parent the deviance from the expected ratios is not alarming. Therefore it can be concluded that the colored phenotype is heritable.

C1	*C2*	*a2-m1(III)**	*Bz1*		*C1*	*c2*	*a2*	*Bz1*
c1	*c2*	*a2*	*bz1*	ON	*c1*	*c2*	*a2*	*bz1*
914038Q					*a2 wx* tester			

Figure 4.3. A cross showing the possible genotypes of *914038Q* and *a2 wx* testers. *C1* and *C2* genes are arbitrarily chosen as the two other genes that are heterozygous in *914038Q*. In fact they could be any two of the three i.e., *C1, C2* and *A1*. The * indicates a putative change in the *a2-m1(III)* allele.

4.3.1.2. Waxy mutable phenotype In the selfed ear the waxy trait segregated as 3 (*Wx* plus *wx-m*) : 1 *wx* and in outcross ear as 1 (*Wx* plus *wx-m*) : 1 *wx* (Table 4.2). The ratios are as expected and therefore it can be concluded that the waxy mutable phenotype of *914038Q* exception also is heritable. In the selfed and outcross ears apart from the expected *wx-m* and *wx* kernels some *Wx* kernels also are segregating (Table 4.2). The percentage of these *Wx* kernels ranged from 23.8% in the selfed ear to about 8.3% in the outcross ear. The *wx-844* allele shows high rates of both somatic and germinal excision. Hence the *Wx* kernels may represent either early somatic excision or

germinal revertants of *wx-844* allele. Therefore the *Wx* kernels should be considered as the derivatives of *wx-844* allele and hence combined with waxy mutable kernels in arriving at the phenotypic ratios. The high percentage of *Wx* kernels in the selfed ear (23.8%) is probably due to high dosage of *wx-844* allele (present in three doses).

4.3.2. Heritability of *914039V*

The plant grown from exception *914039V* is selfed (cross 1 in Table 4.3) and outcrossed onto *a2 wx* tester (cross 2 in Table 4.3). The expected ratios are the same as those of *914038Q* exception. The results are shown in Table 4.3.

4.3.2.1. Colored phenotype The colored phenotype of the exception *914039V* segregated as 27 (Cl+bz) : 37 cl instead of the expected 3 Cl : 1 cl in the selfed ear (Table 4.3). Based on this result it can be concluded that the exception *914039V* is heterozygous for at least three anthocyanin genes. The possible genotype of

Table 4.3. Heritability of the colored waxy mutable phenotype of the *914039V* exception

Cross: 1. $\frac{a2\text{-}m1(III)^*}{a2}$ $\frac{wx\text{-}844}{wx}$ ⊗ 2. $\frac{a2\text{-}m1(III)^*}{a2}$ $\frac{wx\text{-}844}{wx}$ ON $\frac{a2\ wx}{a2\ wx}$

914039V 914039V 914056

Colored non-waxy colorless waxy

#	Cross	Colored			bronze			colorless			Total	χ^2 [b]
		Wx	wx-m	wx	Wx	wx-m	wx	Wx	wx-m	wx		
1.	914039V ⊗	125	46	18	1	-	5	102	32	74	403	*
2.	914039V ON 4056	46	41	20	-	1[a]	1	71	65	170	415	NS

* = Putative mutant of *a2-m1(III)* allele ; a. spotted (flow); b. The ratios tested are - (Cl+bz) : colorless = 27 : 37 in ⊗ ear and 9 : 23 in outcross ear, *Wx : wx-m : wx* = 9 : 3 : 4 in ⊗ ear and 1 : 1: 2 in outcross ear; * = Significant at 5%, NS = Nonsignificant.

the exception *914039V* is shown in Figure 4.4. The source of these recessive alleles is the *a2 wx* tester as explained in section 4.3.1.1. In the outcross ear the ratio is 9 (Cl + bz) : 23 cl (row #2 in Table 4.3). This ratio could be expected if the genotypic constitution of the exception *914039V* and the *a2 wx* tester are as shown in Figure 4.4.

C1	*C2*	*a2-m1(III)**	*Bz1*		*C1*	*C2*	*a2*	*Bz1*
c1	*c2*	*a2*	*(bz1) ?*	ON	*c1*	*c2*	*a2*	*(bz1) ?*
		914039V					*a2 wx* tester	

Figure 4.4. A cross showing the possible genotypes of *914039V* and *a2 wx* testers. *C1* and *C2* genes are arbitrarily chosen as the two other genes that are heterozygous in F1. In fact they could be any two of the three i.e., *C1, C2* and *A1*. The * indicates a putative change in the *a2-m1(III)* allele. The status of *bz1* can not be ascertained from the observed ratios (see Table 4.3).

The appearance of *bz* kernels in the selfed ear (row #1 in Table 4.3) is rather intriguing. The smaller number of bz kernels can simply not be explained by contamination because both female (*914039V* exception) and male (the contaminating source) should contribute a recessive *bz* allele in order to show bronze phenotype. If the exception *914039V* indeed carries a *bz* allele the ratio of Cl to *bz* should be 3 : 1 upon selfing. But the observed ratio is not 3 : 1 in the selfed ear. In fact, the three phenotypes in the selfed ear segregated as 31 Cl : 1 bz : 32 cl (χ^2 is not significant at 5%) indicating that at least three gene pairs are responsible for the observed phenotypic ratio. In the outcross ear (row #2 in Table 4.3) only two out of 415 kernels are *bz*. These may be contaminants (because the *a2 wx* tester might carry a *bz* allele) or may not be contaminants.

Nonetheless the non-appearance of sectored kernel phenotype in both selfed and outcross ears (Table 4.3) assures that the colored phenotype of *914039V* is heritable. The observed deviations from the expected ratios in the case of the exception *914039V* can also be explained by the contribution of recessive alleles from the *a2 wx* parent as in the case of the exception *914038Q.*

4.3.2.2. Waxy mutable phenotype The waxy mutable phenotype of the exception *914039V* is expected to segregate as 3 *wx-m* : 1 *wx* in the selfed ear and 1 *wx-m* : 1 *wx* in the outcross ear. However, this phenotype segregated as 9 *Wx* : 3 *wx-m* : 4 *wx* in the selfed ear and as 1 *Wx* : 1 *wx-m* : 2 *wx* in the outcross ear (Table 4.3). The number of *Wx* kernels is consistently high in both selfed (56.6%) and outcross (28.2%) ears (Table 4.3). Early excision or reversion alone (as described in section 4.3.1.2) can not explain the high frequency of *Wx* kernels observed in these ears. A 9 : 3 : 4 ratio in the selfed ear suggests a dominant epistatic interaction of an 'independent factor' with the *wx-844* allele that increases the mutation of *wx-844* → *Wx*. This independent factor, which enhances mutability, is termed *Mod2* for *Modifier2* because its dominant effect resembles that of the *'Modifier'* element (hereinafter will be denoted as *Mod1*) described by McClintock (1956, 1957, 1958, 1965; see section 2.4.5.4).

On the basis of the observed ratio in the selfed ear, the exception *914039V* is assumed to be heterozygous for *wx-844* and *Mod2.* Based on the dominant epistasis model of *Mod2* and *wx-844*, the observed phenotypes can be explained as follows. Gametes receiving both *wx-844* and *Mod2* would be *Wx*, those receiving *wx-844* without *Mod2* would be waxy mutable and those receiving two *wx* alleles will always be *wx* irrespective of the presence or absence of *Mod2.* The segregation ratio observed in the

outcross ear i.e., 1 : 1 : 2 (row #2 in Table 4.3) also supports this model. The genetic interaction between *wx-844* and *Mod2* is illustrated in Figure 4.5. The further genetic characterization of *Mod2* is described in section 4.4. The appearance of wx-m phenotype in both crosses (Table 4.3) suggests that this phenotype is heritable.

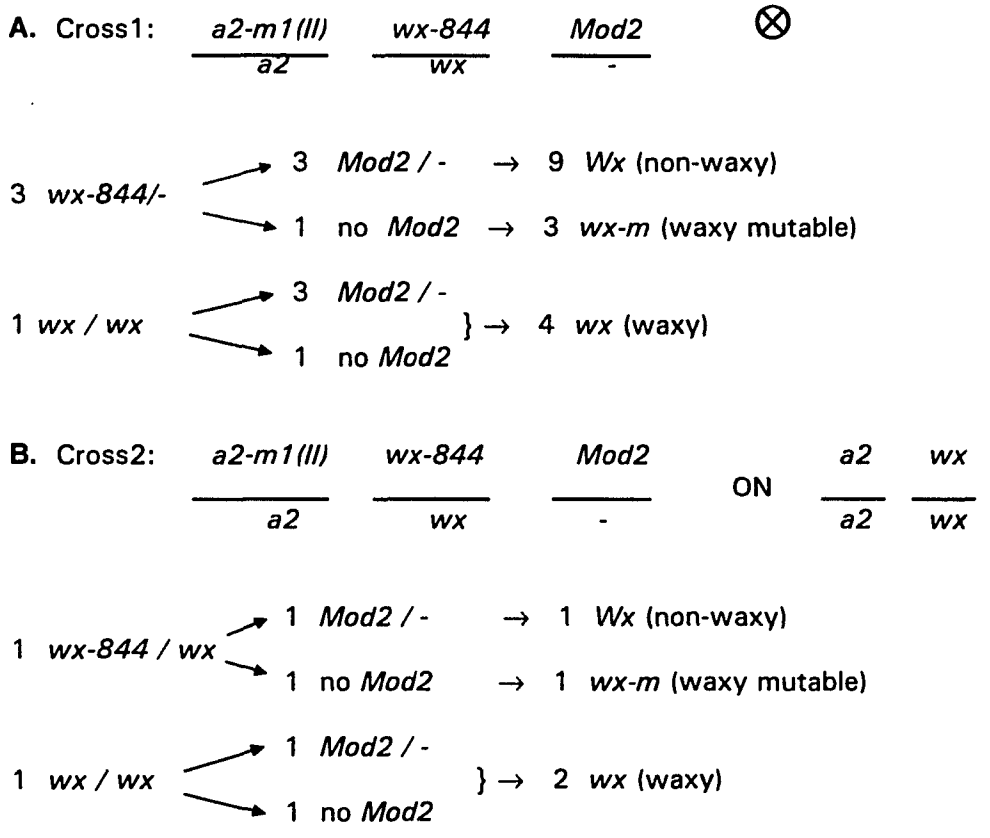


Figure 4.5. Dominant epistasis model for *En1* and *Mod2* interaction. The expected ratios from selfing and outcrossing the plants carrying *Mod2* and *wx-844::En1* allele are shown.

4.3.3. PCR analysis

The DNA is extracted from the leaf tissue of 2-3 week old seedlings grown from the colored waxy mutable kernels. An 800 bp fragment was amplified from the exception 914039V. This fragment was subcloned into pGEM32f(+) vector and sequenced using Sanger's dideoxy sequencing method. The sequence did not reveal the presence of any footprint at the site of insertion. This result suggests that the exception *914039V* probably arose due to contamination by *A2 wx-844* pollen. The possible sources of contamination are the other testers present in the field which carry both *A2* and *wx-844* alleles. Four such testers were present in the field. They are : *P wx-844, a° wx-844, c2 wx-844* and *c2 wx-844 am(r) I-102*. Contamination by *P wx-844* or *a-m(r) I-102* can be easily identifed by segregation of colored pericarp or low waxy mutability respectively in the selfed ears of the two exceptions. But these traits were not observed and hence these two testers may not be the contaminating source. Thus the contaminating pollen must have come from either of the remaining two i.e., *a° wx-844* or *c2 wx-844.*

Alternatively the lack of footprints might be due to precise excision of the *I* element. However, this alternative can not be genetically proved because we did not use a suitable genetic marker linked either to *a2-m1(II)* or *wx-844* in the male parent to rule out the possibility of genetic contamination.

4.4. Genetic Testing of Dominant Epistasis Model of *wx-844* and *Mod2* Interaction

The genetic identification of *Modifier2* is described in section 4.3.2.2. A model for the interaction between *Mod2* and *wx-844* allele is shown in figure 4.5. In order to test this model three types of crosses were made. A sample of 15 kernels from each of the

three phenotypic classes i.e., cl Wx, cl wx-m and cl wx kernels from the selfed ear (see row #1 in Table 4.3), is planted and the plants grown from them are selfed and outcrossed to the *a2 wx* tester to test the heritability of the respective phenotypes. In addition the *Wx* and *wx* kernels also are outcrossed to the *En2* tester. This test will help determine whether *Modifier2* can enhance the low mutator function of the *En2* element. The source of the selected kernel classes and the various tests made to test the dominant epistasis model and to identify the *Mod2* element are outlined in Figure 4.6.

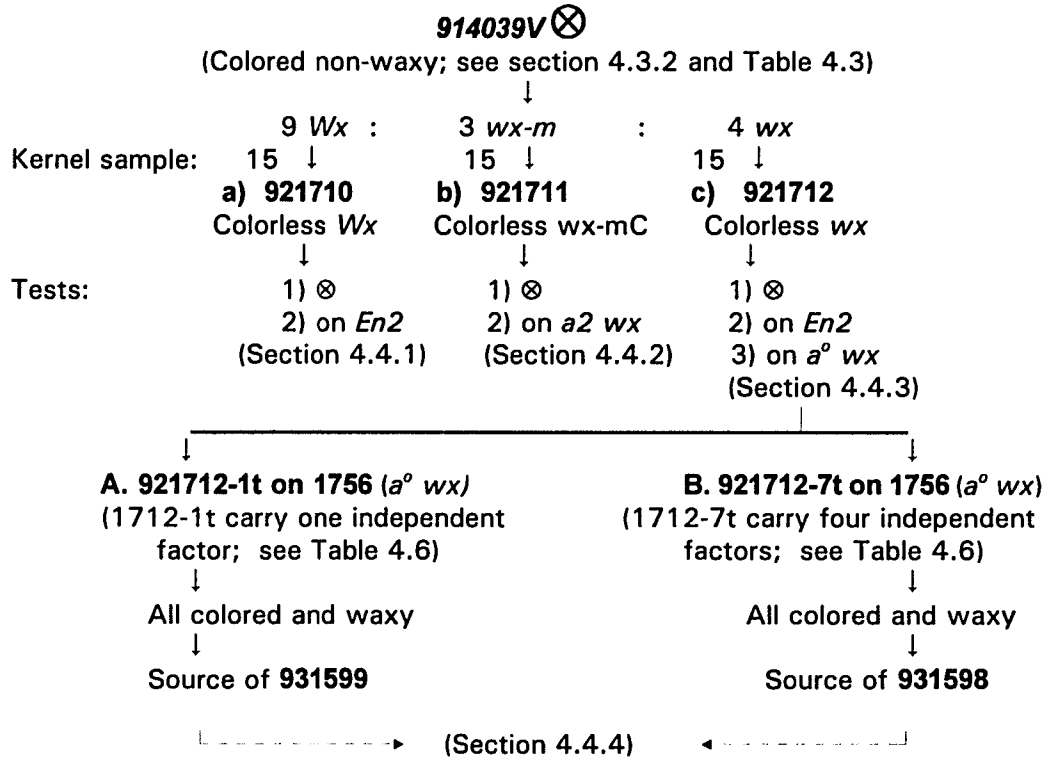


Figure 4.6. Flow diagram showing the outline of various tests made to confirm the dominant epistasis model and the pedigree of colored waxy kernels that are used in testing for the presence of an independently segregating *En* and *Mod* (see section 4.4.4).

4.4.1. Heritability of the Non-waxy (*Wx*) phenotype

According to the dominant epistasis genetic model *Wx* kernels contain both *wx-844* and *Mod2* in homozygous or heterozygous condition (see Fig. 4.5). The possible genotypes of the *Wx* kernels selected from the *914039V* selfed ear (see Table 4.3) are shown in Figure 4.7. When the *Wx* kernels are selfed one would expect to see the appearance of coarse waxy mutability in plants heterozygous for at least the *Mod2* element. The expected phenotypic ratios upon selfing the *Wx* kernels are shown in Figure 4.7.

Of the fifteen *Wx* kernels planted only nine germinated. The genetic constitution is *wx-844/wx* in these nine plants. No coarse waxy mutability appeared in the selfed ears obtained from these plants. A sample of about hundred *Wx* kernels from five out of nine ears were stained by I/KI solution to confirm the *Wx* phenotype. The results are shown in Table 4.4.A.

POSSIBLE GENOTYPES OF *Wx* KERNELS			EXPECTED RATIO
A.	*wx-844* / *wx-844*	*Mod-2* / *Mod-2*	→ All Wx
B.	*wx-844* / *wx*	*Mod-2* / *Mod-2*	→ 3 Wx : 1 wx
C.	*wx-844* / *wx-844*	*Mod-2* / -	→ 3 Wx : 1 wx-m
D.	*wx-844* / *wx*	*Mod-2* / -	→ 9 Wx : 3 wx-m : 4 wx

Figure 4.7. Expected phenotypic ratios upon selfing of colorless *Wx* kernels

Table 4.4. Heritability of the *Wx* phenotype of the kernels carrying *wx-844* and *Mod2*

A. Cross 1: *wx-844* / *wx-844/wx* ⊗ *Mod2* / *Mod2 / -*

#	Cross	CI	colorless			Total	Ratio	χ^2	Stained kernel sample				
			Wx	wx-m	wx				Wx	wx-m C	wx-m L	wx	Sub Total
1	921710-1⊗	-	326	-	98	424	3 : 1	NS	96	1	-	-	97
2	921710-3⊗	-	229	-	58	287	3 : 1	NS	83	3	-	-	86
3	921710-4⊗	-	104	-	24	128	3 : 1	NS	74	30	-	-	104
4	921710-9⊗	-	228	-	52	280	3 : 1	*	100	-	-	-	100
5	921710-12⊗	-	302	-	92	394	3 : 1	NS	97	3	-	-	100

B. Cross 2: *wx-844* / *wx-844/wx* *Mod2* / *Mod2 / -* **ON** *C2* / *c2* *A* / *a* *wx::En2* / *wx*

1	921710-3 on 1758	517	2	1	1	525	-	-	44	9	7	27	87
2	921710-4 on 1758	318	47	27	37	429	-	-	28	25	5	27	85
3	921710-9 on 1758	539	5	7	7	558	-	-	64	18	11	19	112
4	921710-12 on 1757	571	8	1	3	583	-	-	66	14	12	18	110

NS = Non-significant ; * = Siginificant at 5%.

From Table 4.4., it is apparent that coarse waxy mutability did not reappear in all the selfed ears. However, staining revealed that a small fraction (1 to 3%) are indeed waxy mutable in three out of five ears tested. While no waxy mutability appeared in one ear (#4 in Table 4.4.A) their frequency is about 30% in ear #3. The waxy mutability of these kernels does not resemble that of *wx-844* rather it resembles the mutability pattern of *wx-m5* allele (Wessler et al., 1992). The kernels are almost *Wx* looking with one to two very small but distinct *wx* sectors. This result suggests that the *Wx* phenotype might be

due to *Mod2* induced very early excision of *En* from *wx-844* allele in some cases. Thus *Mod2* influences not only the frequency of excision but also the timing of excision. The absence of mutability in the case of ear #4 (Table 4.4.A) might be due to reversion of *wx-844* allele to *Wx* which may or may not be induced by *Mod2*. Nevertheless the occurrence of wx-m kernels suggests that the *En* insert might still be present in the *Wx* locus. In other words the *Wx* kernels still carry *wx-844* allele.

The ability of the *Mod2* to enhance the low mutator function of *En2* also is tested by crossing the *Wx* kernels to *En2* tester (cross2 in Table 4.4.B). If there is no *Mod2* affect on *En2* the expected phenotype of all the kernels from this cross is low (wx → Wx) waxy mutability. If *Mod2* has any enhancing affect on *En2* one would also expect to see coarse (Wx→wx) waxy mutable kernels at varying proportions depending on the number of *Mod2.* No expectations can be made on the colored (or colorless) phenotype that will segregate in the ears obtained from this cross (i.e., cross 2 in Table 4.4.B). This is because the *Mod2* parent (i.e., colorless Wx kernels) carries recessive alleles of some anthocyanin genes (which ones is not determined) either in homozygous or heterozygous condition and the *En2* parent is heterozygous for *C2* and *A1* genes (cross 2; Table 4.4.B).

The results from Table 4.4.B indicate that most of the kernels in these ears are colored except in ear #2. The ¼ th colorless kernels in ear #2 (Table 4.4.B) might be due to heterozygosity of an anthocyanin pathway gene in both male (*Mod2*) and female (*En2*) parents. In the remaining three ears the low frequency of colorless kernels might be due to self contamination of the female parent i.e., *En2* tester.

Since the number of kernels in these ears is very high only a sample of kernels is stained to test the affect of *Mod2* on *En2* (Table 4.4.B). The presence of coarse waxy

mutable kernels indicates that *Mod2* can complement the M function of *En2*. However, this test does not show whether the coarse waxy mutability is due to *Mod2* or *En1* ? Both are supposed to be present in *Wx* kernels (i.e., male parent in cross 2 of Table 4.4.B). It was shown that *Spm-s* can complement *Spm-w* (McClintock, 1958). *Spm-s* and *Spm-w* are analogous to *En1* and *En2* respectively. Hence a different test was performed with the sib *wx* kernels (derived from the same ear as the *Wx* kernels; kernel sample c in Fig. 4.6) to address whether they contain any independently segregating *En1* other than the one present at *wx-844* allele. This test is described in section 4.4.3.

4.4.2. Heritability of the waxy mutable (wx-m) phenotype

The coarse waxy mutable kernel class segregating in the selfed ear of exception *914039-V* (Table 4.3 and kernel sample b in Fig. 4.6) is hypothesized to be due to the presence of the *wx-844* allele and carry no *Mod2* (Fig. 4.5). Fifteen colorless coarse waxy mutable kernels were planted and nine germinated. These nine plants were selfed and outcrossed to *a2 wx* tester. These tests will reveal the nature of coarse waxy mutability with different doses of *wx-844* and in the absence of *Mod2*. Thus these tests will provide data on the frequency of *Wx* kernels in the absence of *Mod2*. The results are shown in Table 4.5.

The coarse waxy mutability is heritable in both selfed and outcross ears. The segregation of the waxy phenotype indicates that they are *wx-844/wx* in constitution except 921711-8 which is *wx-844/wx-844*. The classification of the *Wx* phenotypic classes is not based on kernel staining. The coarse waxy mutability can be clearly seen in the colorless kernels and hence not stained. In the case of outcross ears only the colored kernels are stained. Owing to the triploid nature of the endosperm, the number

Table 4.5. Heritability of coarse waxy mutable kernels from 914039V⊗ ear (see Table 4.3)

A. Cross 1 : *wx-844* / *wx-844 / wx* ⊗ (921711 ; Colorless coarse waxy mutable)

#	Cross	Cl			cl			Total	Ratio tested	χ^2	% of Wx
		Wx	wx-m	wx	Wx	wx-m	wx				
1	921711-1 ⊗	-	-	-	87	119	74	280	3 : 1	NS	33.6
2	921711-2 ⊗	-	-	-	110	162	62	334	3 : 1	**	32.9
3	921711-3 ⊗	-	-	-	134	209	109	452	3 : 1	NS	29.6
4	921711-4 ⊗	-	-	-	258	-	88	346	3 : 1	NS	74.6
5	921711-5 ⊗	-	-	-	101	252	112	465	3 : 1	NS	22.4
6	921711-6 ⊗	-	-	-	81	115	55	251	3 : 1	NS	32.3
7	921711-8 ⊗	-	-	-	143	67	-	210	3 : 1	*	68.1
8	921711-9 ⊗	-	-	-	62	90	51	203	3 : 1	NS	30.5
9	921711-10 ⊗	-	-	-	26	16	14	56	3 : 1	NS	46.4

B. Cross 2 : *wx-844* / *wx-844 / wx* (921711 ; Colorless and coarse waxy mutable) ON *a2* / *a2* *wx* / *wx* (921601-1660, 921803-1825; Colorless and waxy)

1	921711-1 on 1657	-	-	-	34	38	55	127	1 : 1	NS	26.8
2	921711-2 on 1659	40	78	110	28	89	82	427	1 : 1	*	15.9
3	921711-2 on 1820	46	57	87	31	53	75	359	1 : 1	NS	21.4
4	921711-3 on 1659	11	10	25	22	8	29	105	1 : 1	NS	31.4
5	921711-4 on 1621	67	3	88	95	2	90	345	1 : 1	NS	46.9
6	921711-5 on 1657	43	39	72	29	42	70	295	1 : 1	NS	24.4
7	921711-6 on 1820	-	-	-	69	168	217	454	1 : 1	NS	15.2
8	921711-8 on 1654	49	44	-	56	35	-	184	1 : 1	NS	57.1
9	921711-8 on 1659	78	68	-	91	57	2	296	1 : 1	NS	57.1
10	921711-9 on 1659	-	-	-	88	82	163	333	1 : 1	NS	26.4
11	921711-10 on1636	-	-	-	99	57	141	297	1 : 1	NS	33.3

NS = Non-significant ; * = Significant at 5% ; ** = Significant at 1%

of *wx-844* alleles can range from one to three in selfed ears and only one in outcross ears.

The percent of *Wx* kernels is calculated as the proportion of *Wx* kernels among the total number of kernels. The percent of *Wx* kernels ranged from 22.4 to 74.6 in selfed

ears (Table 4.5.A) and 15.2 to 57.1 % in outcross ears (Table 4.5.B). On an average the percent of *Wx* kernels is slightly higher in selfed ears than in outcross ears. In the outcross ears only one dose of *wx-844* is present and hence all the kernels receiving this allele should be coarse waxy mutable. Thus the high proportion of *Wx* kernels in the outcross ears suggest that this phenotype may not be influenced by the dosage of *wx-844* alleles and may be a direct result of the high mutable nature of *wx-844* allele itself.

The frequency of *Wx* kernels in the original colored exception i.e., *914039V*, ranged from 56.6 % in selfed ear and 28.2 % in the outcross ear (see Table 4.3) which are comparable to that observed in the crosses with coarse waxy mutable kernels that lack *Mod2*. Does this mean that the 9 : 3 : 4 ratio in the case of *914039V* exception is only a chance occurrence and can be explained as the property of *wx-844* allele itself without invoking the presence of an independently segregating *Mod2* ? This can be answered by testing the colorless waxy sib kernels (which lack the *wx-844* allele) from the *914039V* selfed ear (kernel sample c in Fig. 4.6) for the presence of any 'modifier' like factors that are capable of altering the mutability of *En/Spm* reporter alleles other than *wx-844*. This test is described in the following section.

4.4.3. Test for the presence of independently segregating factors in the colorless waxy kernels

Fifteen colorless waxy kernels (from *914039V* ⊗ ear; kernel sample c in Fig. 4.6) were planted and fourteen germinated. These 14 plants were crossed onto *En2* and a^o *wx* testers. The crosses with *En2* will reveal whether a *Mod* like element capable of enhancing the low mutability of *En2* is present in the waxy selections or not. The results are shown in Table 4.6. Since the number of kernels in each ear (obtained from crosses

with *En2* tester; see Table 4.6) is very high only a sample of about 100 kernels were stained to determine the type of waxy mutability. The results indicate that there are at least 1 to 5 independently segregating factors (i.e., not linked to *wx* locus) influencing the waxy mutability in these plants.

As mentioned in section 4.4.1 it is not certain whether the increased mutability in these crosses is due to *En1* or due to *Modifier2*. In order to determine the presence of an independently segregating *En1*, colorless waxy selections are outcrossed to a^o *wx* tester. Six ears were obtained and all of them produced colored kernels indicating that the

Table 4.6. Testing for the presence of an independently segregating factor in colorless waxy kernels.

Cross: *wx* / *wx* ± *En1* ± *Mod2* **ON** *C2* *A1* / *c2* *a1* *wx::En2* / *wx*
(921712 ; Colorless waxy) (921757 to 1760 ; Colored, low wx-m)

#	Cross	Colored				Total	Stained kernel sample					# of El.
		Wx	wx-mC	wx-mL	wx		Wx	wx-mC	wx-mL	wx	Total	
1	921712-1 on 1757	7	109	119	201	436	7	109	119	201	436	1
2	921712-2 on 1760	13	185	46	256	500	13	185	46	256	500	2 - 3
3	921712-3 on 1760	-	-	-	-	300	1	23	24	57	105	1
4	921712-4 on 1757	-	-	-	-	300	8	54	3	45	110	4
5	921712-5 on 1760	-	-	-	-	600	10	34	3	64	111	4
6	921712-6 On 1758	-	-	-	-	625	6	51	8	53	118	3
7	921712-7 on 1760	-	-	-	-	575	1	55	4	58	118	4
8	921712-8 on 1760	-	-	-	-	625	-	21	32	64	117	1
9	921712-9 on 1760	-	-	-	-	550	-	16	24	31	71	1
10	921712-10 on 1757	-	-	-	-	525	-	-	42	34	76	0
11	921712-11 on 1758	-	-	-	-	525	6	52	1	64	123	5
12	921712-12 on 1757	-	-	-	-	425	5	25	2	39	71	4
13	921712-13 on 1760	-	-	-	-	450	2	35	4	46	87	3
14	921712-14 on 1757-22	-	-	-	-	425	6	37	1	49	93	5

respective colorless kernel selections (from *914039V*⊗ ; Table 4.3) are *A1/A1*. Plants grown from these colored kernels are subsequently crossed to various *En/Spm* reporter alleles at the *A1* locus. These tests are described in the following section.

4.4.4. Tests to determine whether the independent factors are *En1* and/or *Mod2*

Two rows of colored waxy kernels (12 kernels per row) derived from the ears obtained by crosses listed as A and B in Figure 4.6 were planted in 1993 field. The two rows, numbered 931598 and 931599, are derived from 921712-7 (carry four independent factors; see Table 4.6., row # 7) and 921712-1 (carry one independent factor; see Table 4.6., row # 1) respectively. The pedigree of these colored kernels is shown in Figure 4.6. The plants grown from these kernels are crossed to two categories of testers:

a. Standard *En1* reporter alleles like *a1-m(r), a1-m1 sh Wx* or *a1-m1 Sh wx*. These crosses will reveal the presence of an independently segregating *En1* in the colorless waxy kernels of *914039V* selfed ear.

b. *En1* reporter alleles with low mutability states. For example *a1-m11112, a1-m15719-A1* and *a1 wx::En2* or *a1-m1 wx::En2*. These tests will not only report the presence of *En1* but also help in screening for the presence of *Mod2* by its enhancing affect on the low mutability states of these testers. Since it was shown that *Mod1* affect is revealed only in the presence of *En1* (McClintock, 1956, 1957, 1958), testers like *a1-m11112+En1* and *a1-m15719-A1+En1* also were included which help in testing the *Mod2* affect on these reporter alleles in case the male parent (i.e., plants grown from the colored waxy kernels from rows 931598 and 931599; see Fig. 4.6) carries no *En1*.

The crosses with *a1-m1* are more informative with regard to revealing the presence of *Modifier* element. These results indicated that at least three independent factors, *En1*,

a low-*En* (*En2*-like) and *Mod2,* are present in the colored waxy kernel selections. With regards to the content of *En1, low-En* and *Mod2* the plants grown from colored waxy kernels (i.e., from rows 931598 and 931599) can be divided into four classes: a) plants containing neither *En1* nor *Mod2*, b) plants containing only *En1*, c) plants containing both *En1* and low-*En* and d) plants containing both *En1* and *Mod2* which complement each other to show high kernel spotting (section 4.4.4.1). However, only the results from class d plants i.e., those containing *En1* and *Mod2* are presented here. The results from these plants confirm that the 9 *Wx* : 3 *wx-m* : 4 *wx* ratio observed in the case of *914039V* selfed ear (Table 4.3) is indeed due to the interaction of *Mod2* with *wx-844* allele.

4.4.4.1. Plants carrying *En1* and *Mod2* The various crosses (as described above) made with plants grown from colored waxy mutable kernels (from rows 931598 and 931599) revealed that four of these plants (out of twenty three plants) carry both *En1* and *Mod2* (see Table 4.7). Strong evidence for the presence of both *En1* and *Mod2* in these plants came from crosses with *a1-m1* tester (Table 4.7.A). Ears obtained from this cross showed segregation of high spotted kernels in addition to standard *a1-m1* type spotted kernels (which are included under low spotted column in Table 4.7.A). The high spotting in *a1-m1* crosses ranges from 7-10 b (on average 8-9 b; Fig. 4.8) and resembles *a1-m(r)* spotting. The low spotting ranged from 1-5b + 1-2c and occasional pale sectors (resembles standard *a1-m1* spotting; see Fig. 4.8). The presence of high spotting (higher than standard *a1-m1* spotting) is adequate enough to conclude that these plants carry *Mod2*.

Based on the observed segregation ratios a genetic model that can explain these results can be proposed. The model is based on the following assumptions: low *a1-m1* type spotting is due to *En1*, colorless phenotype is due to *Mod2*, high spotting is due to

Table 4.7. Results from plants containing *En1* and *Mod2*

A) Cross 1: *A* *R* *Mod2* *En1* X *a1-m1 sh*

a *r* - - *a1-m1 sh*

Colored, round and waxy (931598 and 931599) × Colored, shrunken and non-waxy (931615 and 931616)

#	Cross	Cl	Cl+ Sp	Mott	Spotted		cl	Pale	Total	Ratio[c]	χ^2	# of El
					H[a]	L[b]						
1	931598-2t / 1615	50	-	63	23	45	15	7	203	1 : 1	NS	3
2	931598-3.5 / 1616	98	-	75	67	85	5	11	343	1 : 1	NS	4
3	931598-3.5 on 1616	184	-	-	-	172	-	5	361	1 : 1	NS	5
4	931598-8 / 1615	178	-	-	36	37	45	33	339	1 : 1	NS	2
5	931599-1 / 1615	19	55	31	46	56	13	1	221	1 : 1	NS	4

a = 7-10b ; b = 3-5b, some with 1c and pale sectors ;
c = 1(Cl, Cl+Sp, Mott) : 1(Sp, cl, pale)

B) Cross 2: *A* *R* *Mod2* *En1* ON *a-m(r)*

a *r* - - *a-m(r)*

Colored, round and waxy (931598 and 931599) × Colorless, round and non-waxy (931617 and 931618)

6	931598-3.5 on 1618	26	-	-	24[a]	4[b]	-	-	54	1:1[c]	NS	-

a = 7-9b ; b = 3-5b ; c = 1 Cl : 1Sp

C) Cross 3: *A* *R* *Mod2* *En1* ON *a1-m11112*

a *r* - - *a / a1-m11112*

Colored, round and waxy (931598 and 931599) × Pale, round and non-waxy (931585 and 931589)

7	931598-8 on 1585	111	-	-	45[a]	15[b]	-	48	219	1:1[c]	NS	-
8	931599-1t on 1589t	15	8	-	8	15	-	3	49	1:1	NS	-

a = 2-4b+ many pale sectors; b = 2-4b (no pale sectors); c = 1 (Cl, Cl+sp) : 1 (Sp, pale)

Table 4.7. continued

D) Cross 4: *A* *R* *Mod2* *En1* ON *a1-m11112* *En1*

a *r* - - *a / a1-m11112* -

Colored, round and waxy (931598 and 931599) — Low spotted, round and non-waxy (931590 and 931591)

#	Cross	Cl	Cl + Sp	Mott	Spotted		cl	Pale	Total	Ratio[c]	χ^2	# of El
					H[a]	L[b]						
9	931598-2t on 1590	59	-	-	-	59	-	-	118	1 : 1	NS	-
10	931598-3.5 on 1590	173	-	-	-	83	97	-	353	1 : 1	NS	-
11	931598-8 on 1590	229	-	-	14	171	-	4	418	1 : 1	NS	-

a = 2-3b+7-9a ; b = 2-3b ; c = 1 Cl : 1(Sp, cl, Pale)

E) Cross 6: *A* *R* *Mod-2* *En1* ON *a* *wx::En2*

a *r* - - *a* *wx / wx::En2*

Colored, round and waxy (931598 and 931599) — Colorless, round and wx-mL (931609 - 931612)

#	Cross	Colored			Colorless			Total	Ratio[a]	χ^2	#of El
		wx-m C	wx-m L	wx	wx-m C	wx-m L	wx				
12	931599-1t on 1612t	36	54	-	48	57	-	195	1 : 1	NS	-

a = 1Cl : 1cl or 1wx-mC : 1wx-mL

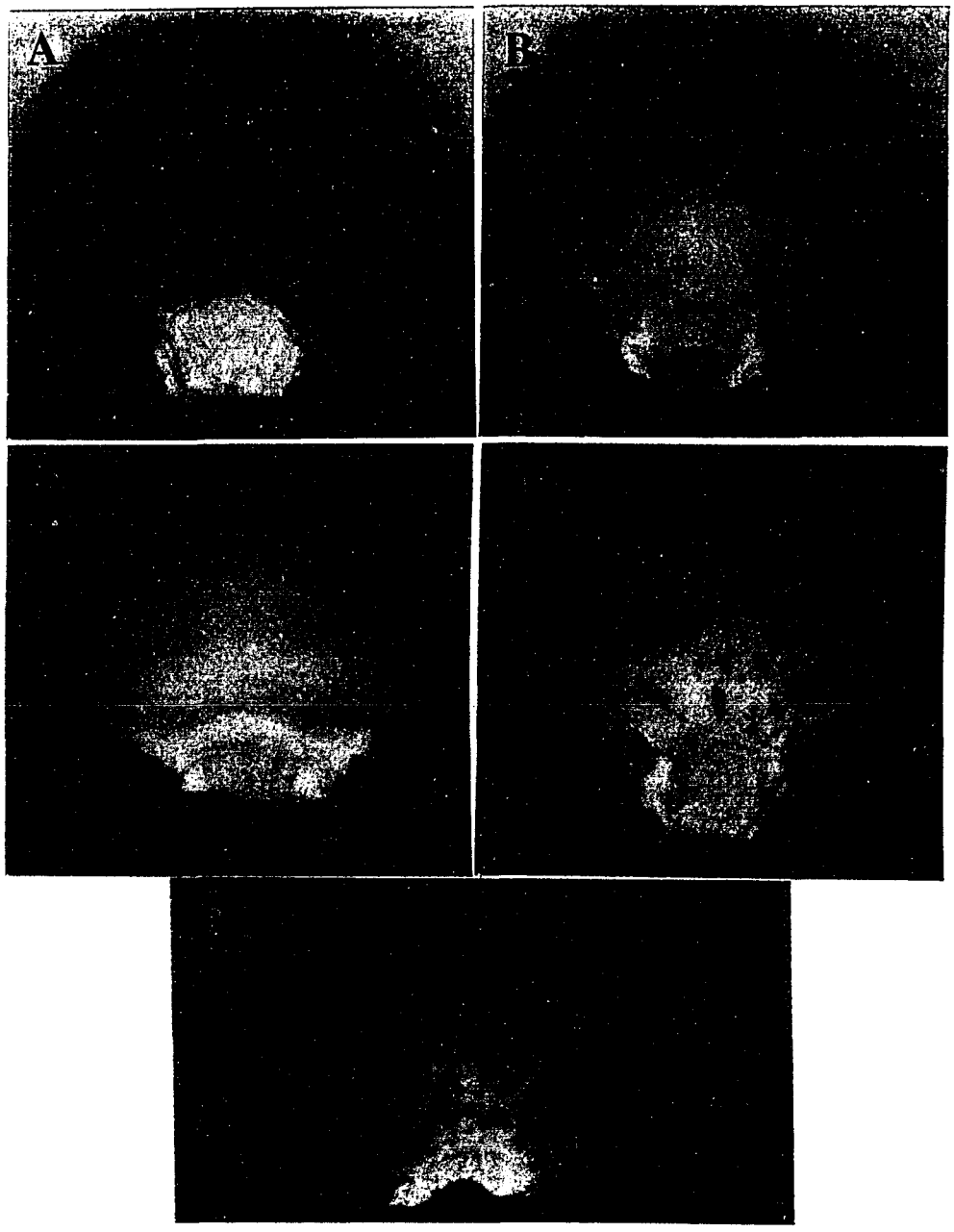


Figure 4.8. Phenotypes observed in an ear segregating for *En1* and *Mod2*. These kernels are from the cross 931598-8/1615. Kernels showing : A. Colored (due to *A1* allele), B. pale (due to *a1-m1* allele; contain neither *En1* nor *Mod2*), C. colorless (supposed to contain *a1-m1* but the colorless phenotype probably suggests suppression of *a1-m1* by *Mod2)*, D. low spotted (due to *En1*) and E. high spotted (due to the interaction of *Mod2* and *En1* with *a1-m1* allele) phenotypes.

interaction of both *En1* and *Mod2* and finally pale kernels are due to absence of both *En1* and *Mod2*. The phenotypes resulting from the interaction between *a1-m1, En1* and *Mod2* are illustrated in Figure 4.9.

Based on the genetic assumptions presented in Figure 4.9., the number of respective elements present in these four plants is determined. The results are shown in Table 4.8. In these four plants the number of *En1* ranges from 1 to 5 and each plant has at least one *Mod2*. The plant 931598-3.5 shows contrasting results when crossed as female and male respectively with *a1-m1* tester (see Tables 4.7.A and 4.8). This plant when crossed as female showed both high spotted (7-10b with no pale or c type sectors) and colorless kernels indicating the presence of at least one *Mod2* (Table 4.8). However, when crossed as male all the spotted kernels are of *a1-m1* type (4-5b + 1-2c, few with pale sectors) and no colorless kernels appeared. These reciprocal differences suggest that the high spotting phenotype may depend on the dosage of *Mod2*. The lack of colorless kernels in the outcross ear also suggests that the S function of *Mod2* is probably

	+ En1	***- En1***
+ Mod2	High Spotting	Colorless(?)
- Mod2	Low Spotting	Pale

Figure 4.9. Genetic model illustrating the interaction of *En1* and *Mod2* in kernels of the genotype *a1-m1/ a1*. The question mark indicates an uncertainity about the colorless phenotype because colorless kernels did not appear when the *Mod2* carrying parent is used as male (Table 4.7.A).

Table 4.8. Estimation of the number of *En1* and *Mod2* in plants carrying both (from cross1, Table 4.7.A)

Serial #	Plant #	Cross-ed as	# of Elements			Phenotypic ratios based on the estimated element number									
						Expected					Observed				
			Total	*En1*	*Mod2*	Cl	Sp.H	Sp.L	cl	Pale	Cl	Sp.H	Sp.L	cl	Pale
1	931598-2	♀	3	2	1	101.5	38.1	38.1	12.7	12.7	113	23	45	15	7[a]
2	931598-3.5	♀	4	3	1	170.5	74.6	74.6	10.7	10.7	173	67	85	5	11[b]
		♂	5	5	-	180.5	-	174.9[c]	-	5.6	184	-	172	-	5[b]
3	931598-8	♀	2	1	1	169.5	42.4	42.4	42.4	42.4	178	36	37	45	33[b]
4	931599-1	♀	4	3	1	110.5	48.3	48.3	6.9	6.9	105	46	56	13	1[a]

a = χ^2 value significant at 5% ; b = Non significant χ^2 value ; c = Only one type of spotting is observed in this cross

dependent on its dosage. In this regard it should be pointed out that McClintock's *Mod1* shows no S function and no dosage effect (McClintock, 1957, 1958). Due to limited data it can not be concluded that *Mod2* is different from *Mod1* by having S function and dosage effect. Therefore more data from reciprocal crosses would be helpful in further characterization of *Mod2* functions.

The results from plant 931598-8 fit perfectly the assumptions made in developing the genetic model for *En1-Mod2* interaction (see Fig. 4.9). The plant 931598-8 is homozygous *RR* and hence no mottled or colorless kernels are expected when this plant was crossed by the *a1-m1* tester. However, colorless kernels appeared and they are thought to be due to the S function of *Mod2*. Hence the kernels obtained from this ear (ear# 4 of Table 4.7.A) are best suited to design various crosses in order to test the assumptions of the genetic model.

Only one of these four plants was crossed to the *a1-m(r)* tester (Table 4.7.B). In the ear obtained from this cross there are no significant differences in the spotting pattern. Most of the spotted kernels are of 7-9 b and there are only four low spotted kernels (3-5b Table 4.7.B). The *I* element of *a1-m(r)* encodes a repressor product namely TNPR (Cuypers et al., 1988) which reduces the frequency of excision. Therefore it can be proposed that the lack of spotting differences might be due to the negation of *Mod2* action by TNPR.

Crosses with *a1-m11112* also yielded two types of spotted kernels (Table 4.7.C). The low spotted kernels are 2-4b with no pale sectors (see panel A in Fig. 4.10). The high kernel spotting is characterized by 2-4b spots plus many pale sectors (see panel B in Fig. 4.10) whose intensity of color is similar to the pale coloration of the *a1-m11112* allele

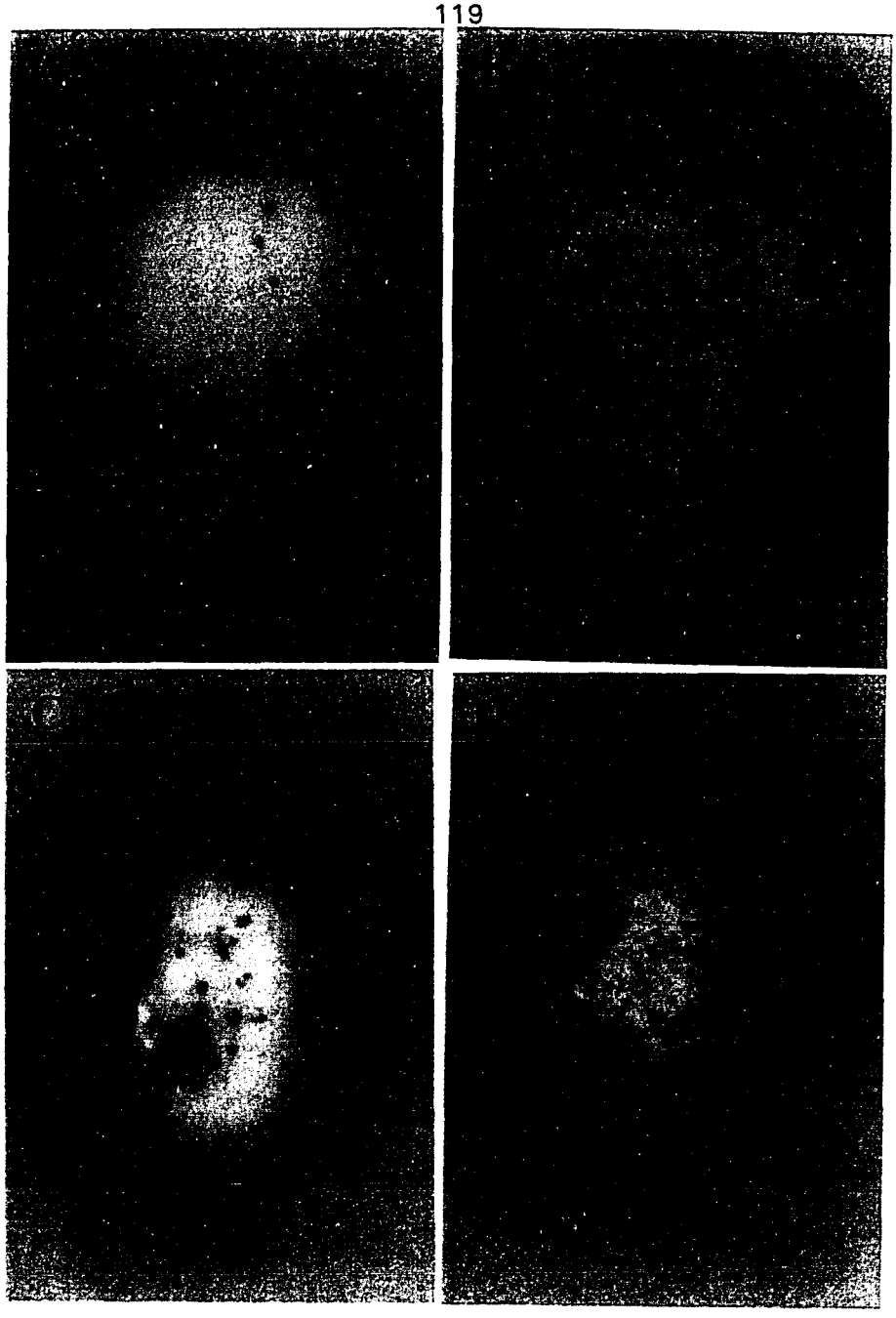


Figure 4.10. Interaction of *Mod2* with *a1-m11112* allele carrying none (A and B) or at least one standard *En1*(C and D). A. Low spotted due to *En1* from the male parent (see cross 3, table 4.7.C); B. Same genotype as in A but shows many pale sectors due to inactivation of *En1*; C. Typical *a1-m11112* spotting in the presence of standard *En1*; D. Increased `a' type spotting and may be due to *Mod2* action.

observed in the absence of *En1*. Therefore the pale sectors could be due to inactivation of *En1* (from male parent; see cross3 in Table 4.7) rather than *Mod2* induced.

When the *Mod2* carrying plants are crossed to the *a1-m11112+En1* tester, the resulting ears produced mainly low spotted kernels (2-3b; Table 4.7.D; Fig. 4.10). Therefore presence of a different standard *En1* did not help either the *En* or *Mod2* (from the male parent) in complementing the low mutable state of *a1-m11112* tester. However, in the case of ear #11 in Table 4.7.D., showed segregation of few high spotted kernels. The 14 kernels shown under high spotting class are different from low spotted kernels with respect to 'a' type spotting i.e., the high spotted kernels are 2-3b+7-9a with many small pale sectors (see panel D in Fig. 4.10) whereas the low spotted kernels are only 2-3b (see panel C in Fig. 4.10). Nevertheless the data are insufficient to demonstrate *Mod2* affect on the low mutability state of *a1-m11112* allele in the presence of a standard *En1* element.

Only one ear was obtained from crosses with the *a1 En2* tester (Table 4.7.E). The proportion of coarse waxy mutable kernels in this one ear suggests that a single element contributed by the male parent is responsible for the increased mutability. However, owing to the paucity of data, it can not be determined whether the increased mutability is due to *En1* or due to *Mod2* (see Tables 4.7.E and 4.8).

In conclusion the various genetic tests described in this section especially the tests with *a1-m1* tester provide evidence for the presence of a modifier element i.e., *Mod2* in the exception *914039V*. The *Mod2* element is responsible for the observed 9*Wx* : 3 *wx-m* : 4 *wx* ratio in the selfed ear of *914039V* (Table 4.3).

5. DISCUSSION

5.1. Stability of the *I* Element at the *a2-m1(II)* Allele

Transposable elements play a role in creating genetic variation that may be useful to the organism in adapting to the adverse environmental conditions such as drought, disease infection etc., (Schwarz-Sommer et al., 1985b; Wessler, 1988). The main source of genetic variation created by transposition activity is the footprints generated at the site of insertion upon element excision (Schwarz-Sommer et al., 1985b; Wessler, 1988). In certain cases the insertion of an element itself can cause genetic variation. In this regard the case of stable inserts is of considerable interest.

Stable inserts can be broadly classified into two types. The first kind are 'truly stable' inserts which may be defined as those *I* elements that are unable to excise from a particular insertion site even in the presence of an active autonomous element. The second type of stable inserts are those where stability of a non-autonomous element is achieved by the absence of an active autonomous element or due to methylation of either the non-autonomous or the autonomous element. However, this kind of stability is only transient because exposure of such elements to an active autonomous element would render them active again (see section 2.7.2.1). The present research is concerned with determining the extent of stability of only the truly stable inserts (derived from actively transposing elements) and not the transiently stable inserts.

As mentioned in section 2.7.1., the stable inserts derived from retrotransposons like *Cin1, Cin4, Stoner* etc., (Schwarz-Sommer et al., 1987b; Blumberg vel Spalve et al., 1990; Varagona et al., 1991) and the insertions belonging to the *Tourist* family of

transposable elements (Bureau and Wessler, 1992) are not considered here for two reasons. First, instability is not a characteristic feature of retroelements whose insertions are stable irrespective of their activity. Second, elements like *Tourist* are not presently active. Therefore the stable inserts considered here are the ones derived from actively transposing progenitor elements like *Ac* and *En/Spm* (reviewed in section 2.7).

Genetic and molecular analysis of the insert at the *a2-m1(III)* allele (Menssen et al., 1990), change of state alleles derived from *bz-m13* allele (Bunkers et al., 1993) and the *d-Spm* insert at *o2-23* allele (Aukerman and Schmidt, 1993) have revealed that an insert can become stably inserted because of two main reasons. These include the large scale loss of the subterminal TNPA binding motifs and the deletion of a few nucleotides within the TIRs (see section 2.7.2.2). Though such deletions render these *I* elements stably inserted some of these inserts continue to interact with the products (especially with TNPA in the case of *En/Spm*) encoded by their respective autonomous elements. These observations led us to question whether these inserts can ever remain stable in the presence of such continued interaction ? The answer to this question also bears upon the extent of stability of the genetic variation created by such stable inserts.

Not much data are available on the extent of stability of the inserts mentioned in the above examples in the presence of their corresponding autonomous elements. The experiments conducted by Neuffer report the stability of two *a1-m* alleles exposed to X-ray and UV radiation only in the absence of the respective autonomous elements (Neuffer, 1966; see also section 2.7.3). Therefore the present study is undertaken with the objective of studying the extent of stability of the type of stable inserts mentioned above in the presence of an active autonomous element.

We have chosen the stable insert of *a2-m1(III)* allele as our experimental material to test the extent of its stability in the presence of an active autonomous *En/Spm* element. The *I* element of the *a2-m1(III)* allele is stably inserted and no evidence of excision either somatic or germinal has been identified by McClintock who first identified and described this allele (McClintock, 1958). Molecular analysis of this *I* element revealed that this *I* element acts as an intron in the intronless *A2* gene and the lack of excision of this element (stable insertion) is thought to be the result of the loss of all the TNPA binding motifs present at its 5' end. Though the effect of the acquisition of an intron on *A2* gene expression has not been tested molecularly, elements of this kind are the potential sources of stable genetic variation. However, the 3' binding motifs of this *I* element continue to interact with the TNPA product of *En/Spm*, thereby leading to the question of how stable this *I* element can be?

From what is known about the molecular structure of the *I* element of *a2-m1(III)* we reasoned that the *I* element can still change its state at a frequency much lower than that observed in the case of elements with intact subterminal regions. Before attempting to design the genetic strategy to find changes of state of *a2-m1(III)* allele, we asked what are the chances that McClintock could not have found such a change? The probable reasons could be, first, the size of *a2-m1(III)* population that she screened might be small. Based on her literature reports (McClintock, 1957, 1958, 1971) we estimate that she must have screened about 50 to 75 thousand kernels. Second, in the majority of her crosses with the *a2-m1(III)* allele, the proportion of colored kernels ranges from one fourth to half. The removal of the *I* element from the transcript leaves behind 21 additional nucleotides in the *A2* transcript. The addition of 7 extra amino acids at this insertion site does not seem to

alter the *A2* function significantly (Menssen et al. 1990). Thus excision events or deletions within the 3' TNPA binding motifs of the *I* element would most likely result in colored exceptions which in McClintock's analysis might have been included in the already segregating colored class.

After due consideration of McClintock's genetic experiments and the recent molecular experiments (Menssen et al., 1990), we have designed a genetic strategy that is based on the following features (see also section 4.1., 4.2 and figure 4.1). The strategy is aimed at screening at least a population of two hundred thousand kernels (about thrice as many screened by McClintock) to find changes of *a2-m1(III)* allele. The genetic strategy that we employed allows us to test for the presence of an active *En* even in the colored kernels. We have used the *wx-844* allele as the source of an autonomous *En*. The *wx-844* allele is the result of insertion of an autonomous *En* in an intron of the *Wx* locus (Peterson, 1985; Periera et al., 1985). Therefore the unstable *Wx* phenotype can be used as a diagnostic tool in determining the presence or absence of *En* even in the colored kernels. In this regard it should be mentioned that in her 1971 report McClintock described the use of the *wx-m8* (non-autonomous) allele in *a2-m1(III)* experiments. This *wx-m8* allele also helps in tracking the presence of *En* in the colored kernels. Again the population carrying both *wx-m8* and *a2-m1(III)* might be very small (about 25,000 kernels). The homozygosity of *a2-m1(III) wx-844* material allows the segregation of a lesser number of colored kernels than that observed in McClintock's crosses. The number of colored kernels in our crosses depend on the rate of germinal reversion of the *wx-844* allele which is rather high. Even after allowing for this high rate of germinal reversion, the size of the colored kernel class observed in our crosses is far less than that observed in McClintock's

crosses. The homozygosity of the *a2-m1(III) wx-844* also renders the F1 genotype uniform. This feature not only reduces the size of the colored kernel class but also maximizes the effective population size that can be screened for the exceptions.

5.1.1. Colored waxy mutable exceptions

As described in section 4.2 the F1 kernels are screened for the presence of colored waxy mutable kernels. This exceptional phenotype is indicative of a putative change in the *a2-m1(III)* allele. These changes could include small nucleotide changes in or deletion of the 3' TNPA binding motifs of the I element. If a deletion extends into the adjacent *A2* gene it may result in completely colorless kernels. However, we did not screen for such colorless kernels for two reasons. First, it is very difficult to distinguish between the colorless exceptions and the colorless waxy mutable kernels resulting from high copy number of *En/Spm* (see the description of *a2-m1(III) wx-844* tester in Table 3.6). Eventhough the expected phenotype of the F1 is coarse sectored and waxy mutable, we often observed a high number of fine sectored and colorless waxy mutable kernels in the F1 ears. We suspect that these patterns of spotting are due to an increase in the copy number of *En/Spm* elements in the male parent. It is possible that in the male parent some inactive or cryptic elements at unlinked sites might have been activated by the trans-active function of the *En/Spm* at *wx-844* allele or due to transposition of *En* away from the *wx-844* allele. Second, since McClintock reported that the *I* element of *a2-m1(III)* is highly stable, we did not initially anticipate large scale deletions at this locus. In addition we are interested in the type of changes where the intron (i.e., the *I* element) is still retained by the *A2* locus. This is because the wildtype *A2* is intronless and acquisition of an intron (i.e., the *I* element insertion) has the potential of altering the *A2* gene expression

significantly. However, the expression of the *a2-m1(III)* allele compared to the wildtype *A2* is not studied in detail. Our search for a mutation of *a2-m1(III)* where the insert is more intron-like (i.e., no interaction with *En* encoded TNPA) than its progenitor would allow us to compare the expression of the wild type *A2* gene that has no intron with the *A2* gene that acquired an intron. Nevertheless the results of our stability experiments and the conclusions we arrived thereupon might be biased in favour of *a2-m1(III)* to *A2* changes and ignores completely *a2-m1(III)* to *a2* changes (see also section 5.1.2).

Apart from the colored waxy mutable kernels, colored non-waxy and colored waxy kernel exceptions could result (see Fig. 4.1 and Table 4.1) from reversion of *wx-844* allele to *Wx* or reversion and simultaneous mutation of *wx-844* to *wx* respectively. In both cases the excised *En* must have been either lost or inactivated because the kernels are colored instead of being sectored. Since the *En* insertion at *wx-844* allele is in the intron (Periera et al., 1985; Klösgen et al., 1986) one should expect to see a high frequency of reversion to *Wx* rather than mutation to *wx*. Thus, the high number of colored waxy kernels (see Table 4.1) might be due to heterozygosity of the *wx-844* allele in some of the male plants which give one half colored waxy kernels in the F1 ears. We did not screen the F1 ears if they showed segregation of more than half colored kernels. However, if an independent *En*, other than the one at the *wx-844* allele, is present in the male parent the number of colored kernels will be less than half. Depending on the number of independent *En* the colored waxy kernels may be ≤ ¼ the total kernels and such ears are included in the screening. Alternatively, inactivation of *En/Spm* element (eventhough the element is inserted in the intron of the *Wx* gene) might be the cause for colored waxy phenotype in some cases if not all. We have some evidence (but no definitive data) to support this

conclusion. McClintock observed a unique phenotype where the borders of the colored and colorless sectors (usually very few) are not sharp but gradually fading in kernels carrying both *a2-m1(III)* and *Spm*. McClintock has ascribed this phenotype as due to the progressive inactivation of the *Spm* element in somatic tissue. We also have observed such phenotypes in our experiments and staining of a sample of such kernels revealed the presence of all three possible *wx* phenotypes i.e., some are *Wx*, some are wx-m and some are *wx*. Therefore inactivation of *En/Spm* present at *wx-844* allele might lead to the *wx* phenotype in certain cases.

Thus, the critical exception is a colored waxy mutable kernel, a kernel that has an *En/Spm* but fails to suppress the *a2-m1(III)* allele. We have identified two colored waxy mutable kernel exceptions (*914038Q* and *914039V*) from the screening of approximately 225,000 F1 kernels (see Fig. 4.1 and Table 4.1) at a frequency of about $1X10^{-5}$.

The colored waxy mutable phenotype of the two exceptions i.e., *914038Q* and *914039V* is heritable (see section 4.3). Though the original phenotype of *914039V* exception is colored non-waxy it is still considered waxy mutable because the waxy mutability phenotype segregated in subsequent heritability tests. A dominant modifier of *En* also is found segregating in this exception (see table 4.3 and section 4.3.2.2). Therefore the original *Wx* phenotype of *914039V* might be only a somatic event probably resulting from the *modifier* induced very early excision of *En* from *wx-844* allele. The oddity of the genetic ratios observed in the heritability tests (table 4.2 and 4.3) does not preclude us from concluding that the colored waxy mutable phenotype of these exceptions is heritable. These complex ratios are the result of the segregation of several recessive alleles of the other anthocyanin pathway genes which are contributed by the *a2-wx* tester.

In these heritability tests one should only be concerned about whether the sectored phenotype reappeared ? and whether the waxy mutable phenotype is segregating ? In the heritability tests with the two exceptions the sectored phenotype never reappeared and waxy mutability always segregated. Therefore the conclusions made on the heritability of colored waxy mutable phenotype are valid.

The dilemma generated by the segregation of the *bz* kernels (some of which are flow-type spotted) in the selfed and outcross ears is not pursued further. A similar *bz*-flow spotted phenotype observed in *bz-rcy-Cy* studies was determined to be due to *Ac*-flow (Schnable, 1986). The spotted kernels that we observed might be due to the *Ac*-flow element also.

The fidelity of the two exceptions could be confirmed, using PCR, by the presence of the *I* element and/or definitive footprints at the *a2* site. The PCR and sequence analysis of the DNA extracted from these two exceptions did not reveal the presence of either the *I* element or the footprint at the insertion site. Therefore these two exceptions are not considered to be due to a putative change in the *a2-m1(II)* allele as initially thought but could be due to genetic contamination by pollen carrying the *A2* and *wx-844* alleles (see section 4.4). Alternatively, these two exceptions could still represent mutations at *a2-m1(II)* and the lack of the *I* element or the footprints might be due to the precise excision of the *I* element. Although precise excision is not common in plants, in maize precise excision was observed in two out of seven somatic revertants analysed in the case of *wx-m8* allele (Schwarz-Sommer et al., 1985b). Apart from maize, two cases of precise excision were reported in the case of *male sterility (MS2)* gene tagging experiment in *Arabidopsis* using *En/Spm* system (Aarts et al., 1993). Study of 12 revertants of *ms2::I*

allele (in total 24 PCR amplified fragments, two from each revertant, were cloned and sequenced) indicated that in two cases namely PA6 and PA46 there are no added or deleted nucleotides surrounding the insertion site and these two sites resembled the wildtype sequence exactly (Aarts et al., 1993). In any case a more ideal experiment would have included a genetic marker linked either to *a2-m1(III)* or *Wx-844* allele in the *a2-m1(III) wx-844* stock to rule out the possibility of genetic contamination. The closest marker that can be used along with the *wx-844* allele is *bz* which is about 25 cM from *wx-844.* The high per cent of crossing over would not be efficient. Another marker that can be used is the *bt* gene linked to the *a2-m1(III)* (7 cM apart). However, the brittle nature of the kernel makes the scoring of waxy mutability difficult while selecting the kernels to be used as a male parent (see Figure 4.1). It is important to select kernels with coarse waxy mutability because this indicates the presence of an active *En* in those kernels. Because of these reasons the *bt* marker is not used.

5.1.2. The *I* element of *a2-m1(II)* is very stable

In the present study our attempts to isolate changes in the state of *I* element of the *a2-m1(II)* allele did not yield any new states among the 225,000 kernels screened. This suggests that the *I* element is not only stably inserted but also highly stable with regards to changing its composition in the presence of an active autonomous *En/Spm* element.

It has been shown that new states arise by deletion of sequences within the elements of the preexisting states. The deletion end points are often bordered by sequences resembling the 12 bp consensus sequence of TNPA binding motif and a short duplication (Tacke et al., 1986; Masson et al., 1987; Schiefelbien et al., 1988; Bunkers et al., 1993). This observation suggests that the TNPA protein might be involved in

causing deletions within the elements. In the case of *a2-m1(III)* apart from the 3' TNPA binding motifs two other binding motifs with partial homology to the binding motif consensus are located in the 5' region, one at the nucleotide position 1009 and the other at the nucleotide position 1310. Yet, it seems these motifs have not been used by TNPA and/or TNPD to cause deletions within the *I* element.

The TNPA binding studies with the *En/Spm* subterminal repetitive regions (both 5' and 3' SRRs) indicate that a single motif can effectively bind TNPA (Gierl et al., 1988a). However, the same study showed that the binding motif present at nucleotide position 1310 of *a2-m1(III)* allele (corresponds to the nucleotide position 19 of *En/Spm*) does not bind TNPA because of a mutation (T to C) at the nucleotide position 10 of the consensus sequence of the TNPA binding motif (Gierl et al., 1988a). The recent studies on the biochemical characterization of TNPA indicates that binding of TNPA to the tail-to-tail oriented binding motifs (a set of them, one in each SRR, are present in the standard *En/Spm* element) forms a compact structure (and therefore forms a more stable complex; Trentman et al., 1993). Since the tail-to-tail oriented motifs are located closer to the TIRs, the binding of TNPA to these motifs is thought to be crucial for element excision (Trentman et al., 1993). Thus in the case of the *I* element of the *a2-m1(III)* allele the lack of excision is thought to be due to the absence of a stable stem-loop structure resulting from the deletion of all the binding motifs (including the tail-to-tail oriented motifs) of the 5' SRR (Menssen et al., 1990; Trentman et al., 1993). From these observations it can be inferred that binding of TNPA to the motifs present at both ends, not just to the motifs present at the 3' end as in the case of *a2-m1(III)* allele, is probably necessary in bringing

about a change in state. Binding of TNPA to both ends probably facilitates recruitment of the TNPD protein.

Recently it has been shown that two *En/Spm* encoded products i.e., TNPA and TNPD are needed for element excision (Frey et al., 1990; Masson et al., 1991). A stem loop structure which is hypothesized to be necessary for element excision (Gierl et al. 1989) is not expected to be formed in the case of the *a2-m1(III)* allele (see Fig. 3.4) because of the loss of 5' binding motifs. Thus the lack of excision in the case of *a2-m1(III)* is due to the inability of TNPD to interact with the terminal inverted repeats which are not in close proximity. Since the occurrence of changes in state is dependent on mutator (TNPD) activity (McClintock, 1955), the non-occurrence of mutations in the state of *a2-m1(III)* might be due to absence of TNPD binding to the *I*-TNPA complex. Thus our data suggests that deletions (whose end points are bordered by sequences similar to TNPA binding motif) in elements that lead to new states are probably mediated by TNPD protein and binding of TNPA alone (to the motifs present at one end) is not sufficient to effect a change in state. If at all, protein-protein interactions (such as between TNPA and TNPD) are involved in causing such deletions they probably play a minor role such as stabilizing the element-transposase complex during excision. However, TNPD is not well characterized biochemically and it is not known whether and how it interacts with TNPA.

5.1.3. Stability related to the function of the *I* element as intron

Molecular analysis of *a2-m1(III)* has revealed that the *I* element of the *a2-m1(III)* allele possesses dinucleotide splice site consensus sequences and is capable of splicing from the *A2* mRNA trascript just like an intron (Menssen et al., 1990). It has been observed (Palmer and Logsdon, 1991) that the *I* element of the *a2-m1(III)* allele is "one of

the most clear-cut examples of a transposon that behaves like an intron". This observation is based on the fact that the sequence surrounding the consensus splice site sequences match very closely with that of plant consensus sequence (see panel C in Fig. 3.4). However, the continued interaction of this *I* element with TNPA which results in suppression of *A2* gene expression does not make it a perfect intron (Menssen et al. 1990). No structural changes of this intron are observed in our screening of about 225,000 kernels which suggests that the newly acquired intron of *A2* is very stable. Therefore the *I* element at *a2-m1(III)* allele will probably evolve into a true intron (i.e., it no longer interacts with the TNPA product of *En/Spm*) at a rate similar to the spontaneous mutation rate. Accumulation of several mutations in the TNPA binding motifs would be necessary to convert this *I* element into a true intron. In this regard the *I* element of *a2-m1*(class I state) allele (which is 2.2 kb in length with intact subterminal regions) would probably evolve into a true intron faster than that of *a2-m1(III)*.

5.1.4. Stability of newly created genetic variation

Stable inserts like the *I* element of the *a2-m1(III)* allele are permanent fixations of the respective host genes. Such stable insertions, when inserted in coding sequences, are capable of creating new genetic variation by altering the structure and expression of their respective host genes. Thus in the present case, in order to relate the results of our stability experiments to the stability of transposable element generated new genetic variation, one should answer whether the *I* element insertion in the case of *a2-m1(III)* allele indeed generated new genetic variation ? McClintock observed that the colored phenotype of *a2-m1(III)* is very much similar to that of wildtype *A2* (McClintock, 1958). Northern analysis indicated that the amount of message is similar in both *A2* and *a2-m1(III)* lines

(Menssen et al., 1990). Does this mean that there is no difference in the expression of *A2* and *a2-m1(III)* ?

Several in-vitro studies in plants have showed that the presence of an intron within the gene increases its expression (Callis et al., 1987; McElroy et al., 1990). Though the exact mechanism by which introns increase gene expression is not known the exonic sequences surrounding the intron seem to influence the level of increase in expression (Leuhersen and Walbot, 1991). It has been hypothesized that the observed increase in gene expression resulting from having an intron might result from increased stability of the message (Callis et al., 1987; Leuhersen and Walbot, 1991). Though conclusive experimental evidence on the stability of message in the *a2-m1(III)* allele compared to the wild type allele is lacking (northern analysis only tells how much message there is and not on the stability of the message), the *a2-m1(III)* allele having acquired an intron might be expressing differently than its wild type counterpart much the same way as maize *Adh-1* (Callis et al., 1987) and rice *actin* genes (McElroy et al., 1990). If this is true the increased genetic fitness of *a2-m1(III)* (and other such alleles generated by stable insertions) might confer some selective advantage. The lack of difference in the phenotype of intronless *A2* and the *a2-m1(III)* allele is probably because the increased level of the *A2* product over a certain threshold level may not have significant effect on color development. Molecular experiments that can test the stability of *A2* message would help to determine the differences in the expression of *A2* and *a2-m1(III)* alleles.

We would also like to state that part of the transposable element generated genetic variation that is observed in the breeding lines (Peterson and Salamini, 1986; Cormack et al. 1988; Lamkey et al. 1991) might be the consequence of insertion of elements of the

type present at *a2-m1(III)* or insertion of elements with small deletions in the TIRs (Schiefelbein et al., 1988; Hehl and Baker, 1989; Healy et al., 1993). Variation resulting from such insertions would be very stable.

5.2. *Modifier2*: a dominant *En/Spm* related factor

5.2.1. Origin of *Modifier2*

During the course of our *a2-m1(III)* stability experiments two colored waxy mutable exceptions were isolated. In one of them ie., *914039V* the waxy phenotypes segregated as 9*Wx* : 3*wx-m* : 4*wx* indicating the presence of an independent factor that induces *wx-m* to *Wx* change. Since the phenotype of this factor is similar to McClintock's *Modifier1* (McClintock, 1957) we named this independent factor *Modifier2 (Mod2)*. The exception *914039V* originated in an ear resulting from crossing *a2 wx* (female parent) with *a2-m1(III) wx-844* stock (male parent). The male parent also is selfed to verify the nature of waxy mutability. The selfed ear obtained from the male parent did not show segregation of 3*Wx* : 1 *wx-m* which would be expected if the male parent contained a single *Modifier2* element (data not shown). This observation suggests that the male parent of *914039V* does not contain *Mod2* (see the cross in Table 4.3). Therefore *Mod2* might have originated in a single male gamete that gave rise to the *914039V* exceptional kernel.

5.2.2. Relation of *Modifier2* with *En/Spm* system

The *Modifier1* described by McClintock has no S or M function individually. However in the presence of *En/Spm* it enhanced the spotting of four different states of the *a1-m1* allele. Based on linkage analysis McClintock concluded that *Mod1* is transposable

(McClintock, 1956, 1957, 1958). The specificity of *Mod1* interaction with *En/Spm* reporter alleles and the ability of *Mod1* to transpose led McClintock to believe that *Mod1* is probably a derivative of the *En/Spm* element (McClintock, 1965). Similarly the low M function of *En-malt,* one of several modifiers that are characterized in Peterson's laboratory, led Peterson to conclude that modifiers probably represent deletion derivatives of the *En/Spm* element (Reddy and Peterson, 1983; Peterson, 1987).

Mod2 is very similar to *Mod1* in not having S or M function individually and in interacting with *En/Spm* to augment its M function. In the present study we did not determine whether *Mod2* is transposable or not. However, based on the specificity of *Mod2* interaction with *En/Spm*, we conclude that *Mod2* indeed is a component of *En/Spm* system.

5.2.3. Mode of action of *Modifier2*

Mod2 effect is evident only in the presence of *En1*. Its affect is more clear on *a1-m1* spotting. In the presence of both *En1* and *Mod2* the spotting is increased by at least two fold compared to the spotting with *En1* alone.

In the presence of *En1*, *Mod2* has no significant affect on the mutability of *a1-m11112* and *a1-m15719A-1* (change of state alleles derived from original *a1-m1* and are characterized by low kernel spotting). The low spotting phenotypes of *a1-m11112* and *a1-m15719A-1* are due to the deletion of (the extent of which varies in these two alleles) TNPA binding motifs (cis-acting elements of transposition) present in the subterminal repetitive region (SRR; see section 2.5.2.3). Therefore the absence of *Mod2* effect in these alleles suggests that *Mod2* probably requires intact SRRs (which is the case in the standard *a1-m1*allele) in order to be effective. This does not necessarily mean that the

Mod2 (or its product) directly interacts with the subterminal regions. Rather the role of intact SRRs may be indirect. The *En/Spm* or *I/dSpm* elements excise efficiently only when there is proper stem-loop formation between the element ends. Binding of TNPA protein to the SRR motifs promotes the stem-loop formation. As the number of TNPA binding motifs decrease lesser and lesser TNPA binds to the SRRs and results in weaker stem-loop formation. Therefore the stability of the stem-loop complex, and not the intactness of SRRs per se, is important for *Mod2* function.

McClintock observed that *Mod1* can increase the spotting frequency of four different *a1-m1* states (McClintock, 1958). At least one of those four states is similar to *a1-m11112*. McClintock describes this state as "showing few relatively late occurring mutations", mutations meaning colored spots. Therefore its spotting frequency might be similar either to *a1-m11112* or intermediate to that of *a1-m11112* and *a1-m15719A-1*. Thus *Mod2* may be different from *Mod1* in not being able to increase the spotting frequency of *a1-m11112* and *a1-m15719A-1* alleles.

Mod1 also is shown to be effective in the presence of *Spm-w* (an autonomous *Spm* with highly reduced M function and analogous to *En2*). Owing to limited data we could not conclude whether *Mod2* is equally effective in the presence of *En2* (see Table 4.7.E., and section 4.4.4.1). In this regard crosses between the kernels carrying only *Mod2* and *a1 En2* tester would be more informative.

5.2.4. Molecular nature of *Modifier2*

Not much is known about the molecular nature of *Mod1* which is yet to be cloned. In the present study we did not find any case where *Mod2* is trapped in an already cloned gene. However, the genetic analysis of *Mod2* gives some clues on its molecular nature.

Based on the observations present in the foregoing section (5.2.3) one can conclude that *Mod2* probably encodes a product. Since *Mod2* action is to enhance the M function of *En1*, the product encoded by *Mod2* is probably a truncated version of TNPD. The ability of *Mod2* to enhance the excision rate of a standard *En/Spm* suggests that there is a limiting factor of the TNPD function under normal conditions. What could the limitation be ?

Though not much is known about the biochemical nature of TNPD, based on its function one can assume that it might consist of at least two domains i.e., a protein binding domain that interacts with TNPA and/or other TNPD molecules and a DNA binding domain that specifically binds the TIRs. The excision domain of TNPD can be viewed as a separate domain or part of the same DNA binding domain. After the element ends form into stem-loop structure by the binding of TNPA the TNPD binding probably is achieved via protein(TNPA)-protein(TNPD) interactions. It is not known how many TNPD molecules are necessary to achieve element excision. Probably at least two TNPD molecules, one at each end, would be a minimum that is necessary. Thus the huge element-TNP complex would probably either limit the number of TNPD molecules that can be bound or it may inflict some steric hindrance on the already bound TNPD molecules so that the excision proceeds only at a certain rate.

The truncated product of *Mod2*, which probably carries both excision and protein binding domains, might be capable of binding the complex over and above the critical number of TNPD molecules that can be allowed to bind or it may be capable of obviating the steric hindrance experienced by normal TNPD molecules. *Mod2* (or *Mod1*) depend on *En/Spm* probably for two reasons: first, it needs TNPA to form the stem-loop and second,

the *Mod2* product probably can not interact with TNPA directly (unlike the standard TNPD molecule) and needs TNPD for promoting its own binding to the complex. This implies that TNPD carries two different protein binding domains one that can bind TNPA and the other that can bind other TNPD molecules. TNPD is encoded by a 6 kb transcript and TNPA is encoded by a 2 kb transcript. Therefore the large size of TNPD can accommodate the various binding domains dicussed above.

5.2.5. *Modifier2* is different from *Mediator*

The *mediator* element is thought to provide some helper function to the *En/Spm* element to achieve excision (at normal rate) of an unique *I* element called *Irma* (Muszynski et al., 1993). As the name suggests it only mediates excision and does not modify (either increase or decrease) the excision like other modifiers. Though *mediator* has no S or M function like some of the modifiers it is different from other modifiers and more so from *Mod2* in its mode of action. While mediator's role is assumed to be stabilizing the stem-loop structure *Mod2* acts to increase the excision rate which concurrently needs stable stem loop structures (see section 5.2.3).

5.3. Types of *En/Spm* derivatives

The *En/Spm* system is perhaps the best characterized system, both genetically and molecularly, among all the known transposable element systems in maize. The initial work of McClintock (1954, 1955, 1956, 1957, 1958) and Peterson (1953, 1960, 1961) has identified two main components of this system i.e., the autonomous *En/Spm* and the non-autonomous *I/d-Spm* elements. While autonomous elements bring about their own transposition, non-autonomous elements depend on autonomous elements for their

transposition. Subsequently the isolation of mediator (Muszynski et al., 1993) attests that at least in one case a third component namely *mediator* is needed for transposition. Though *mediator* is involved in the transposition of *Irma* element it is not known whether *mediator* is derived from *En/Spm* element. However, *mediator* is thought to be a modifier like element (Muszynski et al., 1993).

The past four decades of research on *En/Spm* system suggests that the autonomous *En/Spm* elements are frequently subjected to internal deletions. These deletion end points in most cases are bordered by consensus sequence of TNPA binding motifs. This observation and the results of our stability experiments indicate that such internal deletions are probably mediated by both TNPA and TNPD. It is puzzling to see why *En/Spm* did not develop a protecting mechanism to save itself from the action of its own transposase.

Deletions of the En/Spm element come in a wide variety of sizes starting from the simplest deletions in the TIRs to the largest internal deletions. The CS6 allele derived from *bz-m13* is near the full size of *En* but suffers a deletion within the 5' TIR that renders it stably inserted (Schiefelbein et al., 1988). This element though capable of producing the same quality of TNPA and TNPD as the normal *En1* it can not excise because of the deletion in its TIR. The *En2* (7.1 kb; Gierl et al., 1988b) and *Spm-w* (6.7kb; Masson et al., 1987) elements represent the next class of deletion derivatives. These elements have a normal S function but have only weak M function. The modifier *En-malt* probably falls into this size range i.e., between 6 and 7 kb. The modifiers *Mod1* and *Mod2* probably represent the next size range i.e., 4 to 5 kb. This conclusion is based on the action of modifiers on the M function of *En1* and based on the conjecture that these modifiers

encode a TNPD like protein. The tnpD gene located in the first intron of *En/Spm* is about 4.3 kb in length. The next class of derivatives are represented by the negative repressors like *a1-m(r)I 102* (Cuypers et al., 1988) which is about 3.6 kb in length. The *Restrainer* (Peterson, 1976b, 1978) element may also be of the same size as *I102* i.e., between 3 to 4 kb. The elements less than 3 kb in length are predominantly non-autonomous elements and do not encode any product.

Thus elements that are of 6-8 kb in length retain both S and M functions and thus continue to behave as autonomous elements. Elements which are 3-5 kb long lose both S and M functions but continue to encode a product that gives them the modifier function. These elements probably are non-autonomous and transpose in the presence of *En/Spm*. Most of the non-autonomous elements are less than 3 kb in size and do not possess any S, M or modifier functions. A majority of the stable inserts are of the size range as non-autonomous elements (usually less than 2 kb; see section 2.7. for information on sizes of various stable inserts). The *I* element at CS6 allele though larger may be subjected to progressive internal deletions. The smallest *I* element reported is the one at the *o2-23* allele which is about 168 bp in length and composed entirely of the sequences derived from 3' SRR (Aukerman and Schmidt, 1993). Coincidentally this insert also is found to be stably inserted. These observations tempt us to conclude that elements which are less than 1 kb in size should invariably be stable inserts.

BIBLIOGRAPHY

Aarts MGM, Dirkse WG, Stiekema J, Periera A, 1993. Transposon tagging of a *male sterility* gene in *Arabidopsis*. Nature(London) 363:715-717.

Adhya SL, Shapiro JA, 1969. The galactose operon of *E.Coli* K-12. I. Structural and pleiotropic mutations of the operon. Genetics 62:231-247.

Alleman M, Kermicle JL, 1993. Somatic variegation and germinal mutability reflect the position of transposable element *Dissociation* within the maize *R* gene. Genetics 135:189-203.

Aukerman MJ, Schmidt RJ, 1993. A 168 bp derivative of *Suppressor-mutator/Enhancer* is responsible for the maize *o2-23* mutation. Plant Mol Biol 21:355-362.

Banks J, Fedoroff NV, 1989. Patterns of developmental and heritable change in methylation of the *Suppressor-mutator* transposable element. Dev Genet 10:425-437.

Banks JA, Masson P, Fedoroff NV, 1988. Molecular mechanisms in the developmental regulation of the maize *Suppressor-mutator* transposable element. Genes Devel 2:1364-1380.

Baran G, Echt C, Bureau T, Wessler SR, 1992. Molecular analysis of the *wx-B3* allele indicates that precise excision of the transposable *Ac* element is rare. Genetics 130:377-384.

Barklay PC, Brink RA, 1957. The relationship between *modulator* and *activator* in maize. Proc Natl Acad Sci USA 40:1118-1126.

Barta A, Sommergruber K, Thompson D, Hartmuth K, Matzke MA, Matzke AJM, 1986. The expression of a nopaline synthase-human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. Plant Mol Biol 6:347-357.

Belanger FC, Hepburn AG, 1990. The evolution of CpNpG methylation in plants. J Mol Evol 30:26-35.

Bennetzen JL, Swanson J, Taylor WC, Freeling M, 1984. DNA insertions in the first intron of maize *Adh1* affect message levels: cloning of progenitor and mutant *Adh1* alleles. Proc Natl Acad Sci USA 81:4125-4128.

Bennetzen JL, Springer PS, Cresse AD, Hendrickx M, 1993. Specificity and regulation of the *Mutator* transposable element system in maize. CRC Crit Rev Plant Sci 12:57-95.

Berg DE, Howe MM (eds), 1989. Mobile DNA. Am Soc Microb, Washington DC.

Bird A, 1992. The essentials of DNA methylation. Cell 70:5-8.

Blumberg vel Spalve J, Schwarz-Sommer Zs, Saedler H, Peterson PA, 1990. The *Cin2* and *Cin3* insertion elements of *Zea mays* ssp. *parviglumis*. Maydica 35:151-156.

Boeke, 1989. Transposable elements in *Sacharomyces cereviceae*. In: Berg DE and Howe MM (eds), Mobile DNA. Am Soc Microb, Washington DC, pp 335-374.

Brink RA, Williams E, 1973. Mutable *R-Navajo* alleles of cyclic origin in maize. Genetics 73:273-296.

Brink RA, Nilan RA, 1952. The relation between light variegated and medium variegated pericarp in maize. Genetics 37:519-544.

Brown JJ, Mattes MG, O'Reiley C, Shepherd NS, 1989. Molecular characterization of *rDt,* a maize transposon of the *"Dotted"* controlling element system. Mol Gen Genet 215:239-244.

Brown JWS, 1986. A catalogue of splice junction and putative branch point sequences from plant introns. Nucleic Acids Res 14:9549-9559.

Bunkers G, Nelson OE, Raboy V, 1993. Maize *bronze1:dSpm* insertion mutations that are not fully suppressed by an active *Spm*. Genetics 134:1211-1220.

Bureau TE, Wessler SR, 1992. *Tourist* : A large family of small inverted repeat elements frequently associated with maize genes. Plant Cell 4:1283-1294.

Bureau TE, Wessler SR, 1994. *Stowaway* : A new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants. Plant Cell 6:907-916.

Callis J, Fromm M, Walbot V, 1987. Introns increase gene expression in cultured maize cells. Genes Dev 1:1183-1200.

Cavalier-Smith T, 1978. Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. J Cell Sci 34:247-278.

Cavalier-Smith T, 1985. Selfish DNA and the origins of introns. Nature 315:283-284.

Cavalier-Smith T, 1991. Intron phylogeny: a new hypothesis. Trends Genet 7:145-148.

Cech T, 1990. Self-splicing of group I introns. Annu Rev Biochem 59:543-568.

Chang R-Y, Peterson PA, 1994. Chromosome labelling with transposable elements in maize. Theor Appl Genet 87:650-656.

Chomet P, Lisch D, Hardeman KJ, Chandler VL, Freeling M, 1991. Identification of a regulatory transposon that controls the *Mutator* transposable element system in maize. Genetics 129:261-270.

Chuk G, Robbins T, Nijjar C, Ralston E, Courtney-Gutterson N, Dooner HK, 1993. Tagging and cloning a *Petunia* flower color gene with the maize transposable element *Activator*. Plant Cell 5:371-378.

Coen ES, Carpenter R, 1988. A semi-dominant allele, *niv-525*, acts in trans to inhibit expression of its wild-type in *A.majus*. EMBO J 7:877-883.

Coen ES, Carpenter R, Martin C, 1986. Transposable elements generate novel spatial patterns of gene expression in *Antirrhinum majus*. Cell 47:285-296.

Coen ES, Robbins TP, Almeida J, Hudson A, Carpenter R, 1989. Consequences and mechanism of transposition in *Antirrhinum majus*. In: Berg DE and Howe MM (eds), Mobile DNA. Am Soc Microb, Washington DC, pp 413-436.

Cone KC, Burr FA , Burr B, 1986. Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc Natl Acad Sci USA 83:9631-9635.

Cook D, Fedoroff NV, 1992. Regulation of *Spm* promoter activity by the *Spm*-encoded *tnpA* gene product and DNA methylation. Maize Gen Coop Newslett 66:11-12.

Cormack JB, Peterson PA, 1994. Gene marker loss induced by the transposable element *En*, in maize. Genetics 136:1151-1156.

Cormack JB, Cox DF, Peterson PA, 1988. Presence of the transposable element *Uq* in maize breeding material. Crop Sci 28:941-944.

Courage-Tebbe U, Döring H-P, Fedoroff NV, Starlinger P, 1983. The controlling element *Ds* at the shrunken locus in *Zea mays*: structure of the unstable *sh-m 5933* allele and several revertants. Cell 34:383-393.

Cuypers H, Dash S, Peterson PA, Saedler H, Gierl A, 1988. The defective *En -I102* element encodes a product reducing the mutability of the *En/Spm* transposable element system of *Zea mays*. EMBO J 7:2953-2960.

Dash S, 1991. Study of *En* at the *wx-844* allele: Modifier of *En* excision, weak *En* and transposition of *En*. Ph.D. dissertation, Iowa State University. University Microfilms, Ann Arbor, MI (Diss Abstr 91:26187).

Dash S, Peterson PA, 1989. Chromosome constructs for transposon tagging of desirable genes in different parts of the maize genome. Maydica 34: 247-261.

Dellaporta SL, Wood J, James BH, 1984a. Maize DNA miniprep. In: Malmberg R, Messing J, Sussex I (eds) Molecular biology of plants - A laboratory course manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 36-37.

Dellaporta SL, Chomet PS, Mottinger JP, Wood JA, Yu SM, 1984b. Endogenous transposable elements associated with virus infection in maize. Cold Spring Harbor Symp Quant Biol 49:321-328.

Dempsey E, 1985. Induction of the Activators of the *bz-2m* responding allele. In: Freeling M (ed) Plant genetics. Proceedings of the Third annual ARCO Plant Cell Research Institute-UCLA symposium on biology, held in Keystone, Colorado, April 13-19. Alan R Liss Inc., New York, pp 311-316.

Dennis ES, and Brettell RI, 1990. DNA methylation of maize transposable elements is correlated with activity. Philos Trans R Soc Lond B Biol 326:217-229.

Doolittle , 1978. Genes in pieces: were they ever together? Nature 272:581-582.

Dooner HK, 1980. Regulation of the enzyme UFGT by the controlling element *Ds* in *bz-m4,* an unstable mutant in maize. Cold Spr Harb Symp Quant Biol 45:457-462.

Dooner HK, Belachew A, 1989. Transposition pattern of the maize element *Ac* from the *bz-m2(Ac)* allele. Genetics 122:447-457.

Dooner HK, Robbins T, Jorgensen RA 1991. Genetic and developmental control of anthocyanin biosynthesis. Ann Rev Genet 25:173-199.

Döring H-P, 1989. Tagging genes with maize transposable elements: an overview. Maydica 34:73-88.

Döring H-P, Garber R, Nelsen-Salz B, Tillman E, 1985. Transposable element *Ds* and chromosomal reaarangements. In: Freeling M (ed) Plant genetics. Proceedings of the Third annual ARCO Plant Cell Research Institute-UCLA symposium on biology, held in Keystone, Colorado, April 13-19. Alan R Liss Inc., New York, pp 311-316.

Döring H-P, Nelsen-Salz B, Garber R, and Tillman E, 1989. Double *Ds* elements are involved in specific chromosome breakage. Mol Gen Genet 219:199-305.

Dorit RL, Schoenbach L, Gilbert W, 1990. How big is the universe of exons? Science 250:1377-1382.

Doyle JJ, Doyle JL, 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Emerson RA, 1917. Genetical studies of variegated pericarp in maize. Genetics 2:1-35.

Engels WR, 1989. *P* elements in *Drosophila melanogaster.* In: Berg DE and Howe MM (eds), Mobile DNA. Am Soc Microb, Washington DC, pp 437-484.

English J, Harrison K, Jones JDG, 1993. A genetic analysis of DNA sequence requirements for *Dissociation* state I activity in tobacco. Plant Cell 5:501-514.

Evenari M, 1989. The history of research on White-green variegated plants. The Bot Rev 55:106-139.

Fedoroff NV, 1983. Controlling elements in maize. In: Shapiro J (ed) Mobile genetic elements. Academic Press, New York, pp 1-63.

Fedoroff NV, 1989a. About maize transposable elements and development. Cell 56:181-191.

Fedoroff NV, 1989b. Maize transposable elements. In: Berg DE and Howe MM (eds), Mobile DNA. Am Soc Microb, Washington DC, pp 375-411.

Fedoroff NV, 1989c. The heritable activation of cryptic *Suppressor-mutator* elements by an active element. Genetics 121:591-608.

Fedoroff NV, Banks J, 1988. Is the *Suppressor-mutator* element controlled by a basic developmental regulatory mechanism ? Genetics 120:559-577.

Fedoroff NV, Wessler SR, Shure M, 1983. Isolation of the transposable maize controlling elements *Ac* and *Ds*. Cell 35:235-242.

Fedoroff NV, Furtek D, Nelson Jr OE, 1984. Cloning of the *Bronze* locus in maize by a simple and generalizable procedure using the transposable controlling element *Ac*. Proc Natl Acad Sci USA 81:3825-3829.

Fedoroff NV, Masson P, Banks J, Kingsbury J, 1988. Positive and negative regulation of the *Suppressor-mutator* element. In: Nelson OE (ed) Plant transposable elements, Plenum Press, New York. pp 1-16.

Fincham JRS and Sastry GRK, 1974. Controlling elements in maize. Annu Rev Genet 8:15-50.

Fowler RG, Peterson PA, 1978. An altered state of a specific *En* regulatory element induced in a maize tiller. Genetics 90:761-782.

Freeling M, 1984. Plant transposable elements and insertion sequences. Annu Rev Plant Physiol 35:277-298.

Frey M, Tavantzis SM, Saedler H, 1989. The maize *En-1/Spm* element transposes in Potato. Mol Gen Genet 217:172-177.

Frey M, Reinecke J, Grant S, Saedler H, Gierl A, 1990. Excision of the *En/Spm* transposable element of *Zea mays* requires two element-encoded proteins. EMBO J 12:4037-4044.

Friedeman P, Peterson PA, 1982. The *Uq* controlling element system in maize. Mol Gen Genet 187:19-29.

Galas DJ, Chandler M, 1989. Bacterial insertion sequences. In: Berg DE and Howe MM (eds), Mobile DNA. Am Soc Microb, Washington DC, pp 109-162.

Gerats AGM, Groot SPC, Peterson PA, Schram AW, 1983. Regulation of UFGT activity in the *bz-m4* allele of *Zea mays*: a possible case of gene fusion. Mol Gen Genet 190:1-4.

Gierl A, 1990. How maize transposable elements escape negative selection. Trends in Genet 6:155-158.

Gierl A, Schwarz-Sommer Zs, Saedler H, 1985. Molecular interactions between the components of the *En-I* transposable element system of *Zea mays*. EMBO J 4:579-583.

Gierl A, Lütticke S, Saedler H, 1988a. TnpA product encoded by the transposable element *En-1* of *Zea mays* is a DNA binding protein. EMBO J 7:4045-4053.

Gierl A, Cuypers H, Lütticke S, Periera A, Schwarz-Sommer Zs, Dash S, Peterson PA, Saedler H, 1988b. Structure and function of the *En/Spm* transposable element system of *Zea mays*: Identification of the suppressor component of *En*. In: Nelson OE Jr (ed) Plant transposable elements. Plenum Press, New York, pp 115-119.

Gierl A, Saedler H, Peterson PA, 1989. Maize transposable elements. Annu Rev Genet 23:71-85.

Gilbert W, 1978. Why genes in pieces? Nature 271:501.

Gonella JA, Peterson PA, 1975. The presence of *En* among some maize lines from Mexico, Colombia, Bolivia and Venezuela. Maize Genet Coop Newslett 49:73-74

Gonella JA, Peterson PA, 1977. Controlling elements in a tribal maize from Columbia: *Fcu*, a two unit system. Genetics 85:629-645.

Goodall GJ, Filipowicz W, 1989. The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. Cell 58:473-483.

Goodall GJ, Filipowicz W, 1990. The minimum length of pre-mRNA introns in monocots and dicots. Plant Mol Biol 14:727-733.

Goodall GJ, Filipowicz W, 1991. Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants. EMBO J 10:2635-2644.

Goodall GJ, Kiss T, Filipowicz W, 1991. Nuclear RNA splicing and small nuclear RNAs and their genes in higher plants. Oxford Surveys Plant Mol Cell Biol 7:255-296.

Goodwin TW, Mercer EI, 1983. Introduction to plant biochemistry (2nd ed.). Pergamon Press, New York.

Grant SR, Gierl A, Saedler H, 1990. *En/Spm* encoded tnpA protein requires a specific target sequence for suppression. EMBO J 9:2029-2035.

Griffiths AJF, Miller JH, Suzuki DT, Lewontin RC, Gelbart WM, 1993. An introduction to genetic analysis (5th ed). WH Freeman & Company, New York.

Gruenbaum Y, Naveh-Many T, Cedar H, Razin A, 1981. Sequence specificity of methylation in higher plant DNA. Nature (London) 292:860-862.

Gupta M, Shepherd NS, Bertram I, Saedler H, 1984. Repetitive sequences and their organization on genomic clones of *Zea mays*. EMBO J 3:133-139.

Hartings H, Lazzaroni N, Spilmont C, Asperti L, Di Fonzo N, Thompson R, Salamini F, Motto M, 1990. Molecular analysis of the *Bg-rbg* transposable element system. maize Gen Coop Newslett 64:25.

Healy J, Corr C, DeYoung J, Baker B, 1993. Linked and unlinked transposition of a genetically marked *Dissociation* element in transgenic tomato. Genetics 134:571-584.

Hehl R, Baker B, 1989. Induced transposition of *Ds* by a stable *Ac* in crosses of transgenic tobacco plants. Mol Gen Genet 217:53-59.

Hershberger RJ, Warren CA, Walbot V, 1991. Mutator activity in maize correlates with the presence and expression of the *Mu* transposable element *Mu9*. Proc Natl Acad Sci USA 88:10198-10202.

Jayaram C, Peterson PA, 1990. Anthocyanin pigmentation and transposable elements in maize aleurone. In: Janick J (ed) Plant Breeding Reviews, Vol 8. Timber press Inc, Portland, OR, pp 91-137.

Johns MA, Mottinger J, Freeling M, 1985. A low copy number *Copia*-like transposon in maize. EMBO J 4:1093-1102.

Jordan E, Saedler H, Starlinger P, 1964. 0° and strong polar mutations in the gal operon are insertions. Mol Gen Genet 102:353-363.

Katinakis P, Verma DPS, 1985. *Nodulin-24* gene of soybean codes for a peptide of the peribacteroid membrane and was generated by tandem duplication of a sequence resembling an insertion element. Proc Natl Acad Sci USA 82:4157-4161.

Khedarnath S, Brink RA, 1958. Transposition and the stability of *modulator* in maize. Genetics 43:695-704.

Kiesselbach TA, 1980. The structure and reproduction of corn. University of Nebraska Press, Lincoln, Nebraska.

Kim H-Y, Schiefelbein JW, Raboy V, Furtek DB, Nelson Jr OE, 1987. RNA splicing permits expression of the maize gene with a defective *Suppressor-mutator* transposable element in an exon. Proc Natl Acad Sci USA 84:5863-5867.

Kirk JTO, Tilney-Basset RAE, 1978. The plastids. Their chemistry, structure, growth and inheritance, 2nd ed. Elsevier, Amsterdam.

Klösgen RB, Gierl A, Schwarz-Sommer Zs, Saedler H, 1986. Molecular analysis of the *waxy* locus of *Zea mays*. Mol Gen Genet 203:237-244.

Kunze R, Starlinger P, 1989. The putative transposase of transposable element *Ac* from *Zea mays* L interacts with subterminal sequences of *Ac*. EMBO J 8:3177-3185.

Lamb P, McKnight SL, 1991. Diversity and specificity in transcriptional regulation: the benefits of heterotypic dimerization. Trends Biochem Sci 16:417-422.

Lambowitz AM, 1989. Infectious introns. Cell 56:323-326.

Lamkey KR, Peterson PA, Hallauer AR, 1991. Frequency of the transposable element *Uq* in Iowa stiff stalk synthetic maize populations. Genet Res Camb 57:1-9.

Leu JY, Sun Jh, Lai Y-K, Chen J, 1992. A maize cryptic *Ac*-homologous sequence derived from an *Activator* transposable element does not transpose. Mol Gen Genet 233:411-418.

Leuhersen KR, Walbot V, 1991. Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. Mol Gen Genet 225:81-93.

Lister C, Jackson D, Martin C, 1993. Transposon-induced inversion in *Antirrhinum* modifies *nivea* gene expression to give a novel flower color pattern under the control of *Cycloidea*^{radialis}. Plant Cell 5:1541-1553.

Long D, Martin M, Sundberg E, Swinburne J, Puangsomlee P, Coupland G, 1993. The maize transposable element system *Ac/Ds* as a mutagen in *Arabidopsis*: Identification of an *Albino* mutation induced by *Ds* insertion. Proc Natl Acad Sci USA 90:10370-10374.

Maddaloni M, Ponziani G, Di Fonzo N, Salamini F, Thompson R, Motto M, 1989. Molecular properties of the *wx-m32* allele: a *Bg*-induced unstable mutation. Maize Genet Coop Newslett 63:30.

Maniatis T, Fritsch EF, Sambrook J, 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Masson P, Fedoroff NV, 1989. Mobility of the *Suppressor-mutator* element in transgenic tobacco cells. Proc Natl Acad Sci USA 86:2219-2223.

Masson P, Surosky R, Kingsbury JA, Fedoroff NV, 1987. Genetic and molecular analysis of the *Spm*-dependent *a-m2* alleles of the maize *a* locus. Genetics 177:117-137.

Masson P, Rutherford G, Banks J, Fedoroff NV, 1989. Essential large transcripts of the maize *Spm* transposable element are generated by alternative splicing. Cell 58:755-766.

Masson P, Strem M, Fedoroff NV, 1991. The tnpA and tnpD gene products of the *Spm* element are required for transposition in tobacco. Plant Cell 3:73-85.

McClintock B, 1938. The fusion of broken ends of sister half-chromatids following chromatid breakage at meiotic anaphase. Missouri Agric Exp Sta Res Bull 290:1-48.

McClintock B, 1939. The behaviour in successive nuclear divisions of a chromosome broken at meiosis. Proc Natl Acad Sci USA 25:405-416.

McClintock B, 1941. The stability of broken ends of chromosomes in *Zea mays*. Genetics 26:234-282.

McClintock B, 1942. The fusion of broken ends of chromosomes following nuclear fusion. Proc Natl Acad Sci USA 28:458-463.

McClintock B, 1945. Cytogenetic studies of maize and *Neurospora*. Carnegie Inst Wash Year Book 44:108-112.

McClintock B, 1946. Maize genetics. Carnegie Inst of Wash Year Book 45:176-186.

McClintock B, 1947. Cytogenetic studies of maize and Neurospora. Carnegie Inst Wash Year Book 46:146-152.

McClintock B, 1948. Mutable loci in maize. Carnegie Inst of Wash Year Book 47:155-169.

McClintock B, 1949. Mutable loci in maize. Carnegie Inst Wash Year Book 48:142-154.

McClintock B, 1950. The origin and behaviour of mutable loci in maize. Proc Natl Acad Sci 36:344-355.

McClintock B, 1951. Chromosome organization and genic expression. Cold Spr Harb Symp Quant Biol 16:13-47.

McClintock B, 1952. Mutable loci in maize. Carnegie Inst of Wash Year Book 51:212-219.

McClintock B, 1953. Mutable loci in maize. Carnegie Inst of Wash Year Book 52:227-237.

McClintock B, 1954. Mutations in maize and chromosomal aberrations in *Neurospora*. Carnegie Inst of Wash Year Book 53:254-260.

McClintock B, 1955. Controlled mutation in maize. Carnegie Inst of Wash Year Book 54:245-255.

McClintock B, 1956. Mutation in maize. Carnegie Inst Wash Year Book 55:323-332.

McClintock B, 1957. Genetic and Cytogenetic studies in maize. Carnegie Inst of Wash Year Book 56:393-401.

McClintock B, 1958. The *Suppressor-mutator* system of control of gene action in maize. Carnegie Inst of Wash Year Book 57:415-429.

McClintock B, 1959. Genetic and cytological studies of maize. Carnegie Inst of Wash Year Book 58:452-456.

McClintock B, 1961. Some parallels between gene control systems in maize and bacteria. Am Nat 95:265-277.

McClintock B, 1962. Topographical relations between elements of controlling systems in maize. Carnegie Inst Wash Year Book 61:448-461.

McClintock B, 1963. Further studies of gene-control systems in maize. Carnegie Inst Wash Year Book 62:486-493.

McClintock B, 1964. Aspects of gene regulation in maize. Carnegie Inst Wash Year Book 63:592-602.

McClintock B, 1965a. The components of action of the regulators *Spm* and *Ac*. Carnegie Inst Wash Year Book 64:527-536.

McClintock B, 1965b. The control of gene action in maize. Brookhaven Symp Biol 18:162-184.

McClintock B, 1967. Regulation of pattern of gene expression by controlling elements in maize. Carnegie Inst Wash Year Book 65:568-578.

McClintock B, 1968. The states of a gene locus in maize. Carnegie Inst Wash Year Book 66:664-672.

McClintock B, 1971. The contribution of one component of a control system to versatility of gene expression. Carnegie Inst of Wash Year Book 70:5-17.

McClintock B, 1984. The significance of responses of the genome to challenge. Science 226:792-801.

McElroy D, Zhang W, Cao J, Wu R, 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163-171.

Menssen A, Höhmann S, Martin W, Schnable PS, Peterson PA, Saedler H, Gierl A, 1990. The *En/Spm* transposable element of *Zea mays* contains splice sites at the terminii generating a novel intron from a *dSpm* element in the *A2* gene. EMBO J 9:3051-3057.

Menssen A, Saedler H, Gierl A, 1991. Does *A2* encode a dioxygenase? Maize Gen Coop Newslett 65:50-51.

Montanelli C, Di Fonzo N, Marotta R, Motto M, Soave C, Salamini F, 1984. Occurrence and behaviour of the components of the *o2-m(r)-Bg* system of maize controlling elements. Mol Gen Genet 197:209-218.

Müller-Neumann M, Yoder JI, Starlinger P, 1984. The DNA sequence of the transposable element *Ac* of *Zea mays* L. Mol Gen Genet 198:19-24.

Muszynski MG, Gierl A, Peterson PA, 1993. Genetic and molecular analysis of a three-component transposable element system in maize. Mol Gen Genet 237:105-112.

Nelson Jr OE, Klein AS, 1984. The characterization of an *Spm*-controlled *bronze*-mutable allele in maize. Genetics 106:769-779.

Neuffer MG, 1966. Stability of the suppressor element in two mutator systems at the *A* locus in maize. Genetics 53:541-549.

Nevers P, Saedler H, 1977. Transposable genetic elements as agents of gene instability and chromosome rearrangements. Nature 268:109-115.

Nevers P, Shepherd NS, Saedler H, 1986. Plant transposable elements. Adv Bot Res 12:103-203.

Nowick EM, Peterson PA, 1981. Transposition of the enhancer controlling element system in maize. Mol Gen Genet 183:440-448.

Orton ER, Brink RA, 1966. Reconstitution of the variegated pericarp allele in maize by return of *Modulator* to the *P* locus. Genetics 53:7-16.

Palmer JD, Logsdon JM, 1991. The recent origins of Introns. Current Opin Genet Dev 1:470-477.

Periera A, Saedler H, 1989. Transpositional behaviour of the maize *En/Spm* element in transgenic tobacco. EMBO J 8:1315-1321.

Periera A, Schwarz-Sommer Zs, Gierl A, Bertram I, Peterson PA, Saedler H, 1985. Genetic and molecular analysis of the *Enhancer (En)* transposable element system of *Zea mays*. EMBO J 4:17-23.

Periera A, Heinrich C, Gierl A, Schwarz-Sommer Zs, Saedler H, 1986. Molecular analysis of the *En/Spm* transposable element system of *Zea mays*. EMBO J 5:835-841.

Perlman PS, Butow RA, 1989. Mobile introns and intron-encoded proteins. Science 246:1106-1109.

Peterson PA, 1953. A mutable pale green locus in maize. Genetics 38:682-683.

Peterson PA, 1958. The effect of temperature on the mutation rate of a mutable locus in maize. J Heredity 49:121-124.

Peterson PA, 1960. The pale green mutable system in maize. Genetics 45:115-133.

Peterson PA, 1961. Mutable *a1* of the *En* system in maize. Genetics 46:759-771.

Peterson PA, 1965. A relation between the *Spm* and *En* control systems in maize. Am Nat 99:391-398.

Peterson PA, 1966. Phase variation of regulatory elements in maize. Genetics 54:249-266.

Peterson PA, 1970a. The *En* mutable element system in maize. III. Transposition associated with mutational events. Theor Appl Genet 40:367-377.

Peterson PA, 1970b. Controlling elements and mutable loci in maize: their relationship to bacterial episomes. Genetica 41:33-56.

Peterson PA, 1976a. Changes in state following transposition of a regulatory element of enhancer sytem in maize. Genetics 84:469-483.

Peterson PA, 1976b. Basis for the diversity of states of controlling elements in maize. Mol Gen Genet 149:5-21.

Peterson PA, 1977. The position hypothesis for controlling elements in maize. In: Bukhari AI, Shapiro JA, Adhya S (eds) DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 429-435.

Peterson PA, 1978. Controlling elements: the induction of mutability at the *A2* and *C* loci in maize. In: Walden DB (ed) Maize breeding and genetics, John Wiley & Sons, New York, pp 601-631.

Peterson PA, 1981. Instability among the components of a regulatory element transposon in maize. Cold Spr Harb Symp Quant Biol 45:447-456.

Peterson PA, 1985a. The *Enhancer (En)* system: A maize mobile element system. In: Freeling M (ed) Plant genetics. Proceedings of the Third annual ARCO Plant Cell Research Institute-UCLA symposium on biology, held in Keystone, Colorado, April 13-19. Alan R Liss Inc., New York, pp 369-381.

Peterson PA, 1985b. Virus induced mutations in maize: on the nature of stress- induction of unstable loci. Genet Res 46:207-217.

Peterson PA, 1985c. The isolation of *En1* in the *wx-844* allele. Maize Genet Coop Newslett 59:3.

Peterson PA, 1987. Mobile elements in plants. CRC Crit Rev Plant Sci 6:105-208.

Peterson PA, 1988. The *En* mobile element system in maize. In: Nelson Jr OE (ed) Plant transposable elements. Plenum Press, New York, pp 43-68.

Peterson PA, 1990. *c2-m85-2* gives rise to lethal derivatives. Maize Genet Coop Newslett 64:7

Peterson PA, Salamini F, 1986. A search for active mobile elements in the Iowa stiff stalk synthetic maize population and some derivatives. Maydica 31:163-172.

Pisabarro AB, Martin WF, Peterson PA, Saedler H, Gierl A, 1991. Molecular analysis of the *Ubiquitous (Uq)* transposable element system of *Zea mays*. Mol Gen Genet 230:201-208.

Pohlman RF, Fedoroff NV, Messing J, 1984. The nucleotide sequence of the maize controlling element *Activator*. Cell 37:635-643.

Qin M, Robertson DS, Ellingboe AH, 1991. Cloning of the Mutator transposable element *MuA2*, a putative regulator of somatic mutability of the *a1-Mum2* allele in maize. Genetics 129:845-854.

Raboy V, KimH-Y, Schiefelbein JW, Nelson OE, 1989. Deletions in a *d-Spm* insert in a maize *bronze-1* allele alter RNA processing and gene expression. Genetics 122:695-703.

Raina R, Cook D, Fedoroff NV, 1993. Maize *Spm* transposable element has an enhancer-insensitive promoter. Proc Natl Acad Sci USA 90:6355-6359.

Reddy AR, Peterson PA, 1976. Germinal derivatives of the *En* controlling element system in maize: Characterization of colored, pale and colorless derivatives of *a2-m*. Theor Appl Genet 48:269-278.

Reddy AR, Peterson PA, 1983. Transposable elements of maize : genetic basis of pattern differentiation of some mutable *c* alleles of the *Enhancer* system. Mol Gen Genet 192:21-31.

Reddy LV, Peterson PA, 1984. Enhancer transposable element induced changes at the *A* locus in maize: the *a-m1 6078* allele. Mol Gen Genet 194:124-137.

Reddy LV, Peterson PA, 1985. *Spm* and *I* element changes with the *a-m2 8004* allele in maize. Mol Gen Genet 200:211-219.

Rhoades MM, 1936. The effect of varying gene dosage on aleurone colour in maize. J Genet 33:347-354.

Rhoades MM, 1938. Effect of the *Dt* gene on the mutability of the *a1* allele of maize. Genetics 23:377-397.

Rhoades MM, Dempsey E, 1982. The induction of mutable systems in plants with the high-loss mechanism. Maize Genet Coop Newslett 56:21-26.

Rhodes PR, Vodkin LO, 1988. Organization of the *Tgm* family of elements in soybean. Genetics 120:597-604.

Rio DC, 1990. Molecular mechanisms regulating *Drosophila P* element transposition. Annu Rev Genet 24:543-578.

Robertson DS, 1978. characterization of a *Mutator* system in maize. Mutat Res 51:21-28.

Robertson DS, Stinard PS, 1987. Genetic evidence of *Mutator*-induced deletions in the short arm of chromosome 9 of maize. Genetics 115:353-361.

Robertson DS, Stinard PS, Maguire MP, 1994. Genetic evidence of *Mutator*-induced deletions in the short arm of chromosome 9 of maize. II. *wd* deletions. Genetics 136:1143-1149.

Saedler H, Nevers P, 1984. Transposition in plants: a molecular model. EMBO J 4:585-590.

Saedler H, Starlinger P, 1967. O^0 mutations in the Galactose operon in *E. Coli.* II Physiological characterization. Mol Gen Genet 100:190-202.

Saedler H, Schwarz-Sommer Zs, Gierl A, 1985. The role of plant transposable elements in gene evolution. In: Freeling M (ed) Plant genetics. Proceedings of the Third annual ARCO Plant Cell Research Institute-UCLA symposium on biology, held in Keystone, Colorado, April 13-19. Alan R Liss Inc., New York, pp 271-281.

Salamini F, 1980. Genetic instability at the *opaque-2* locus of maize. Mol Gen Genet 179:497-507.

Sanger F, Nicklen S, Coulsen AR, 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Schiefelbein JW, Raboy V, Fedoroff NV, Nelson Jr OE 1985. Deletions within a defective *Suppressor-mutator* element in maize affect the frequency and developmental timing of its excision from the *bronze* locus. Proc Natl Acad Sci USA 82:4783-4787.

Schiefelbein JW, Raboy V, Kim H-Y, Nelson OE, 1988. Molecular characterization of *Suppressor-mutator* (*Spm*) - induced mutations at the *bronze-1* locus in maize. In: Plant transposable elements (Nelson OE Jr ed). New York: Plenum Press; 261-278.

Schläppi M, Smith D, and Fedoroff NV, 1993. TnpA trans-activates methylated maize *Suppressor-mutator* transposable elements in transgenic tobacco. Genetics 133:1009-1021.

Schnable PS, 1986. Genetic characterization of the *Cy* transposable element system at the *Bz* locus of *Zea mays* L., Ph.D. Thesis, Iowa State University, Ames, IA.

Schnable PS, Peterson PA, 1986. Distribution of genetically active *Cy* transposable elements among diverse maize lines. Maydica 31:59-81.

Schnable PS, Peterson PA, Saedler H , 1989. The *bz-rcy* allele of the *Cy* transposable element system of *Zea mays* contains *a Mu*-like element insertion. Mol Gen Genet 217:459-463.

Schwarz-Sommer Zs, Gierl A, Klösgen RB, Wienand U, Peterson PA, Saedler H, 1984. The *Spm (En)* transposable element controls the excision of a 2-Kb DNA insert at the *wx-m8* allele of *Zea mays*. EMBO J 3:1021-1028.

Schwarz-Sommer Zs, Gierl A, Berntgen R, Saedler H, 1985a. Sequence comparison of "states" of *a1-m1*suggests a model of *Spm(En)* action. EMBO J 4:2439-2443.

Schwarz-Sommer Zs, Gierl A, Cuyper s H, Peterson PA, Saedler H, 1985b. Transposable elements generate the sequence diversity needed in evolution. EMBO J 4:591-597.

Schwarz-Sommer Zs, Shepherd N, Tacke E, Gierl A, Rohde W, Leclercq L, Mattes M, Berndtgen R, Peterson PA, Saedler H, 1987a. Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. EMBO J 6:287-294.

Schwarz-Sommer Zs, Leclercq L, Göbel E, Saedler H, 1987b. *Cin4*, an insert altering the structure of the *A1* gene in *Zea mays*, exhibits properties of nonviral retrotransposons. EMBO J 6:3873-3880.

Sharp PA, 1987. Splicing of messenger RNA precursors. Science 235:766-771.

Shepherd NS, 1988. Transposable elements and gene tagging. In: Shaw CH (ed) Plant molecular biology - A practical approach. IRL Press, London, pp 187-219.

Shepherd NS, Schwarz-Sommer Zs, Blumberg vel Spalve J, Gupta M, Wienand U, Saedler H, 1984. Similarity of the *Cin1* repetitive family of *Zea mays* to eukaryotic transposable elements. Nature 307:185-187.

Shepherd NS, Rhoades MM, Dempsey E, 1989. Genetic and molecular characterization of *a-Mrh-Mrh*, a new mutable system of *Zea mays*. Dev Genet 10:507-519.

Simon R, Starlinger P, 1987. Transposable element *Ds2* of *Zea mays* influences polyadenylation and splice site selection. Mol Gen Genet 209:198-199.

Sommer H, Bonas U, Saedler H, 1988. Transposition-induced alterations in the promoter region affect transcription of the chalcone synthase gene of *A. majus*. Mol Gen Genet 211:49-55.S

Sprague GF, McKinney HH, 1966. Aberrant ratio: an anomaly in maize associated with virus infection. Genetics 54:1287-1296.

Starlinger P, Courage U, Döring H-P, Frommer W-B, Kunze R, Laird A, Merchelbach A, Müller-Neumann M, Tillman E, Werr W, Yoder J, 1985. Plant transposable elements - Factors in the evolution of the maize genome? In: Freeling M (ed) Plant genetics. Proceedings of the Third annual ARCO Plant Cell Research Institute-UCLA symposium on biology, held in Keystone, Colorado, April 13-19. Alan R Liss Inc., New York, pp 251-270.

Sundaresan V, Freeling M, 1987. An extrachromosomal form of the *Mu* transposons in maize. Proc Natl Acad Sci USA 84:4924-4928.

Tacke E, Schwarz-Sommer Zs, Peterson PA, and Saedler H, 1986. Molecular analysis of states of the *A1* locus of *Zea mays*. Maydica 31:83-91.

Taylor AL, 1963. Bacteriophage induced mutation in *Escherchia coli*. Proc Natl Acad Sci USA 50:1043-1051.

Taylor LP, Walbot V, 1985. A deletion adjacent to the maize transposable element *Mu1* accompanies loss of *Adh1* expression. EMBO J 4:869-876.

Trentman SM, Saedler H, Gierl A, 1993. The transposable element *En/Spm* -encoded TNPA protein contains a DNA binding and a dimerization domain. Mol Gen Genet 238:201-208.

van-Schaik NW, Brink RA, 1959. Transpositions of *Modulator*, a component of the variegated pericarp allele in maize. Genetics 44:725-738.

Varagona M, Wessler SR, 1990. Implications for the cis-requirements for *Ds* transposition based on the sequence of the *wx-B4 Ds* element. Mol Gen Genet 220:414-418.

Varagona M, Purugganan M, Wessler SR, 1991. Alternative splicing induced by insertion of retrotransposons into the maize *waxy* gene. Plant Cell 4:811-820.

Voytas DF, Cummings MP, Konieczny A, Ausubel FM, Rodermel SR, 1992. *Copia*-like retrotransposons are ubiquitous among plants. Proc Natl Acad Sci USA 89:7124-7128.

Walbot V, 1991. The Mutator transposable element family of maize. In: Setlow JK (ed) Genetic engineering, vol. 13, Plenum Press, New York, pp 1-37.

Walbot V, Cullis CA, 1985. Rapid genomic change in higher plants. Annu Rev Plant Physiol 36:367-396.

Weil CF, Wessler SR, 1990. The effects of plant transposable element insertion on transcription initiation and RNA processing. Annu Rev Plant Physiol 41:527-552.

Weil CF, Wessler SR, 1993. Molecular evidence that chromosome breakage by *Ds* elements is caused by aberrant transposition. Plant Cell 5:515-522.

Weil CF, Marillonnet S, Burr B, Wessler SR, 1992. Changes in state of the *Wx-m5* allele of maize are due to intragenic transposition of *Ds*. Genetics 130:175-185.

Wessler SR, 1988. Phenotypic diversity mediated by the maize transposable elements *Ac* and *Spm*. Science 242:399-405

Wessler SR, 1989. The splicing of maize transposable elements from pre-mRNA - a minireview. Gene 82:127-133.

Wessler SR, 1991. The maize transposable *Ds1* element is alternatively spliced from exon sequences. Mol Cell Biol 11:6192-6196.

Wessler SR, Varagona MJ, 1985. Molecular basis of mutations at the *waxy* locus of maize: correlation with the fine structure genetic map. Proc Natl Acad Sci USA 82:4177-4181.

Wessler SR, Baran G, Varagona M, Dellaporta S, 1986. Excision of *Ds* produces *waxy* proteins with a range of enzymatic activities. EMBO J 5:2427-2332.

Wessler SR, Baran G, Varagona M, 1987. The maize transposable element *Ds* is spliced from RNA. Science 237:916-918.

Weydemann U, Wienand U, Niesbach-Klösgen U, Peterson PA, Saedler H, 1988. Cloning of the transposable element *Mpi1* from *c2-m3*. Maize Gen Coop Newslett 62:25.

ACKNOWLEDGMENTS

During my stay at ISU I met several nice people who helped me in many ways to make my stay a pleasant experience. Though I felt a great deal of gratitude for their help in the past I might not have made a concerted effort to convey them my heartfelt feelings. So I take this opportunity to thank one and all who helped me a lot both morally and financially.

I thank my major professor Dr. Peterson for giving me a great opportunity to work in his laboratory and for introducing me to maize genetics. I am truly indebted to him for being instrumental in getting me the `Pioneer Hi-Bred Scholarship' at a time when I desperately needed financial support. I thank him for his initiative in arranging me an assistantship through the Department of Agronomy during the last two semesters. I treasure the cytological experience I gained while assisting in his Agronomy 529 class.

I thank my committee members Dr. Hallauer (I always wondered how he can be so cheerful all the time. I wish I could be like him), Dr. Palmer (I appreciate him for not getting angry at me even when I did not return his book `Collection of McClintock's Works' which I borrowed from him a year ago. This book is a great help to me), Dr. Girton (He is one among those nicest professors who always made me feel comfortable talking to him. As TA I gained a valuble experience in his genetics class and if I ever get an opportunity to teach I would follow his teaching style) and Dr. Thornburg (I greatly cherish the molecular experience that I gained while working as a rotation student in his lab. I thank him for giving me the opportunity to work in his lab) for agreeing to serve on my committee.

I sincerely thank Dr. Enger, Chairman of Zoology and Genetics Department, for providing me with the Teaching Assistantship inspite of my belated request. It helped me not only financially but also in gaining a valuble teaching experience. I thank Dr. Cantrel, Head of Agronomy Department for providing me with the assistantship during the last two semesters. I thank Dr. Stadler, Chairman of IGM program, for her constant encouragement and advice.

I thank Dr. Imsande, Dr. Shoemaker and Dr. Schnable for allowing me to work in their respective labs as a rotation student which helped me learn various techniques. I also thank Dr Myers for introducing me to the GCG program, Dr. Larson, Dr. Voytas, Dr. Allen Miller, Dr. Shoemaker, and Dr. Gierl (Munich, Germany) for allowing me to use some of their lab facilities to carryout the PCR experiments of my research.

I thank Dr. John Pierce, Dr. Howard Hershey, Dr. James Wong and Dr. Todd Jones of E.I.duPont deNemours and Company, Wilmington, Delaware, for giving me the summer studentship and for teaching me various plant transformation techniques.

I sincerely acknowledge the great help rendered to me by Gaylan Crim, research assistant of Dr. Peterson, in many aspects of my lab, greenhouse and field experiments.

Ina Couture and Roberta Kerkove of Agronomy Department, Mary Johnson and Eileen Muff of Department of Zoology and Genetics and Linda Wild of IGM program helped me immensely whenever I needed their help. They always greeted me with their enthusiastic and smiling demeanor whenever I approached them with a problem. I thank them all from the bottom of my heart.

I express my indebtedness to two of my colleagues Mr. Eberson Calvo and Mr. Dinesh Kumar for their generous help in my PCR experiments. Over the years my family

of friends grew vastly at ISU. Manzoor Ali Mohideen and Ruying Chang are more than friends to me. They are very helpful to me and I thank them for lending me their compassionate ear. I sincerely thank my colleagues Sudhansu Dash, Mike Muszynski, Etienne Kaszas, Kim Hagemann and Yang Bao Pan for helping me in my research. I will cherish their companionship for a long time. My association with Elizabeth, Rashid, Ruth Kabitzke, Tom Horejsi, Milton Haar, Mohan Bangalore and Beom Seok Seo is truly wonderful. I thank my long time friends Shri Koti Reddy, Pawan Kumar, Udaya Bhaskara Reddy, Chandrasekhar and Brahmanandam for their cheerful letters and moral support.

I thank our family friend and a relative Shri Subbarami Reddy for supporting me financially during the first one and half years of my stay at ISU. Eventhough I repaid his interest-free loan I ever remain indebted to him for his generous gesture which means a lot to me.

I am truly greatful to my uncle Shri Yellamanda Reddy and my grandfather Shri Raghava Reddy for getting me interested in Agricultural Sciences early in my childhood. I thank my wonderful parents for everything they have done to me. I thank my brothers, sisters, uncles and cousins for wishing me well all the time.

Finally, I thank the Pioneer Hi-Bred International for the Scholarship, Departments of Zoology and Genetics and Agronomy for their financial support and for giving me the opportunity to continue my education. I thank the Department of Plant Pathology for letting me use their wonderful computer facility. Last but not least THANK YOU **I S U.**